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HEALTH EFFECTS RESEARCH ON DIMETHYL SULFOXIDE (DMSO) MUNITION RECRYSTALLIZATION PROCESS SOLVENT: PHASE II

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FINAL REPORT

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Laboratory for Energy-Related Health Research
University of California, Davis
Davis, CA 95616-5224

30 June 1988

Supported by:

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Frederick, MD 21701-5010

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In Phase I, the recrystallization process solvent DMSO was found to be mutagenic. In Phase II, several genotoxic tests were used to confirm the mutagenic potential. Attempts to identify the mutagen(s) were not successful. The solvent test substance was already several years old when received. The mutagenic activity, measured in the Ames test, continually declined. The solvent was fractionated and an active fraction was obtained. The active fraction did not contain any of the primary munitions. An attempt at high resolution mass spectrometry was performed at Lawrence Livermore National Laboratory but the active agent(s) could not be identified. A surrogate munitions mixture was prepared using reagent grade DMSO. This mixture was tested in the Ames Assay and was not found to be mutagenic. The mutagenic activity was either a process contaminant, munition breakdown product or an artifact added during the early storage history of the sample.

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FOREWORD

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EXECUTIVE SUMMARY

Methods for separation of munitions components by high pressure liquid chromatography were established. This chromatographic method was used to fractionate the DMSO process solvent used in the recrystallization of munitions into four aliquots for mutagenicity testing. Mutagenic response of a late eluting fraction with Ames tester strains was 2-5 times background; similar responses were observed with unfractionated munitions recrystallization solvent. The mutagenic activity of the active fraction was positive in the bacterial assay (Ames) and confirmed in mammalian assays using both the mouse lymphoma forward mutation assay and the Chinese hamster ovary cell sister chromatid exchange assay. Further attempts to provide a highly pure sample with defined mutagenic activity for mass spectral analysis failed. Age of the sample was likely a factor in decreasing mutagenic activity which, in turn, thwarted attempts to identify the mutagenic component(s). The likely instability of the sample is consistent with the findings that S9 bioactivation is not necessary for mutagenic activity in the Ames assay and that the response observed with evaporator reprocess solvent in the mouse lymphoma assay at the end of the study was only 25% of that observed when this material was tested initially. Mutagenic activity could not be attributed to any of the known components of the reprocess solvent nor was a surrogate mixture prepared by mixing known components of the evaporator solvent found to be mutagenic. Although the active agent was not identified, the mutagenic testing was performed following Good Laboratory Practice guidelines.

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INTRODUCTION

PHASE I - SUMMARY

A pilot plant process developing a new explosive including RDX and HMX munitions uses the solvent dimethylsulfoxide (DMSO) during a recrystallization process and in the production stream it uses some byproduct solvent and sludge material. The well known dermal properties of DMSO, i.e. its efficient absorption through the skin, raised the question of assessing the potential hazard of DMSO as a carrying agent to workers that may be exposed to constituent products that might be dissolved in the DMSO from the munitions process stream. In 1983 our laboratory was asked to evaluate the potential for mutagenicity. In a mammalian cell test system, i.e. the scope of the early research using the mouse lymphoma cells (L5178Y) for the detection of specific locus mutation, was to evaluate possible mutagens contained in the DMSO solvent. In the first phase of the research, three samples, virgin DMSO, recycled solvent, and evaporator sludge were evaluated for mutagenic activity with and without liver S9 activation.

It was found that two DMSO solvent samples containing munition constituents contained a direct acting mutagen(s). Two samples tested of DMSO recycled solvent samples had more acute mutagenic activity than DMSO evaporator sludge sample at comparable dilution of a stock solution. The mutagenic activity in these samples was not augmented with the addition of S9 activation through rat liver S9 microsomal preparations. Since these samples were a mixture of several compounds, the identity of the specific compounds responsible for the mutagenic activity was not known. Independently, Sauers et al., 1983, in evaluating the same material had detected mutagenic activity only in evaporator sludge and not in recycle solvent by means of the Ames Salmonella mutagenic assay. The process involved HMX, RDX, TAX, and SEX plus traces of some stabilizers. Independent testing has shown that these four primary compounds were not mutagenic and thus the Phase I studies ended with the suggestion that either an intermediate breakdown product or some other contaminant was responsible for the mutagenic response in the bacterial and mammalian assay systems (Parmer et al., 1985). It was decided that additional research was necessary and a Phase II research project was planned and executed.

PHASE II

It is this Phase II research that is the subject of this report. The Phase II research plan was designed to verify the presence of the mutagenic activity in bacterial and mammalian assay systems. Both the solvent and the sludge would be evaluated. The systems to be employed in testing included the Ames assay using at least four strains. The mouse lymphoma forward mutation mammalian assay, a mammalian cell transformation assay, and a cytogenetic evaluation using a sister chromatid exchange test was used to examine the potential genetic toxicity of the material. In addition to testing the samples provided, attempts would be made to analyticaly separate components of the samples and to identify the compound or compounds with mutagenic activity. A biodirectional analytical procedure would be used relying heavily upon HPLC (high performance liquid chromatography) for fractionation, and biological testing using the Ames assay. The final task in this phase was to attempt to reconstitute a surrogate of the tested sludge and run a parallel set of tests to determine whether interaction between the pure munition compounds might responsible for the positive genotoxic findings.

BACKGROUND INFORMATION

The samples evaluated at our laboratory were originally produced at Holston Army Ammunition Plant in about 1980. They had been stored for some time under specific conditions unknown to us for some two years prior to our obtaining them. We are unaware of the specifics of production, storage, transfer, and transport to us prior to the samples arriving at our laboratory. The HMX and RDX were received from the Contracting Officer's Representative, Dr. Jack Dacre at U.S. Army Biomedical Research and Development Laboratory (USABRDL). The SEX and TAX were received from SRI International in Palo Alto, CA. All samples were stored in the dark between 18 and 20°C.

MATERIALS AND METHODS

HIGH-PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

All chromatography was performed on an IBM HPLC equipped with a variable wavelength UV detector. Reverse-phase C18 columns (Varian Associates, 0.6 x 25 cm or 1.2 x 25 cm) were used for all separations. HPLC-grade solvents were purchased from Fisher Scientific. Details on the analysis of the specific fractions and subfractions are presented in the figure legends and the results section of this report.

The various fractions and mixtures were evaluated for potential genotoxicity by the use of the Ames assay which is both economical, rapid and sensitive. The Ames mutagenesis assay is based on the use of strains of the bacterium Salmonella typhimurium which have a mutation resulting in the inability to synthesize histidine in the growth medium. Agents to be tested and evaluated are added to the bacteria in the presence or absence of a liver microsomal preparation from rats (S9). This S9 preparation contains enzymes which can convert many promutagens to mutagens thus increasing the capability of the assay to identify a broader spectrum of mutagens. The bacteria in the presence of the test compound on histidine-deficient agar plates are incubated for 48 hours at 37°C and the colonies are counted. The colonies represent the progeny of a single bacterium which has "reverted", i.e. regained the ability to synthesize histidine (going from his - to his +). Strains of TA1535 and TA100 can be reverted (or back mutated) by agents causing base pair exchanges, while TA98, TA1537, and TA1538 are reverted by agents which delete base pairs thus causing a frame shift mutation.

A standard component in this test is the use of positive control for strains that do or do not require activation. Anthramine (2AA) obtained from Sigma Chemical Co. and was used as a positive control for all strains requiring metabolic activation. Aroclor obtained from Litton Bionetics was used to induce rat liver enzymes for the S9 fraction. 2-Nitrofluorene (2NF) was obtained from Aldrich Chemical Co. used as a positive control for strains TA98 and TA1538. Sodium azide (NaAZ) was used a positive control for strains TA100 and TA1535 and 9-aminoacridine (9AA) obtained from Sigma Chemical Co. was used a positive control for TA1537. Control carcinogens used were 2-aminofluorene (2AF) from Sigma Chemical Co. and benzo(a)pyrene (BaP) from Aldrich Chemical Co. The agent grade dimethylsulfoxide (DMSO) was used for the positive controls as a solvent. Primary tester strains TA97, TA100, TA98, TA1535, and TA1537 were obtained from the laboratory of Professor Bruce Ames at the University of California at Berkeley. The primary cultures of tester strains were stored at -60°C in a Revco freezer.

The standard assay utilizes the pour plate method. In this procedure for each plate (Falcon), 2 ml of molten top agar (0.6% agar [Difco] + 10% 0.5mM histidine [Calbiochem]-biotin [Sigma] solution) was mixed with 0.1 ml of a 12 hour culture of bacteria containing approximately 10^8 cells, 0.1 ml of a test agent in phosphate buffered saline (PBS, Gibco), then 0.1 ml of S9 mixture with or without 10% by volume of S9 microsomal preparation (Litton Bionetics) in a 13 x 100 ml culture tube. The contents were thoroughly mixed, then poured onto a minimal-glucose agar plate (0.5% agar with 2% glucose). After solidification of the top agar on a level surface, the plates were incubated for 48 hours at 37°C. The number of revertant colonies were then counted with an automated colony counter (Biotran II). The quality of the background of minute colonies was also noted. All handling of the material to be tested and plates were performed in a biohazard hood with 100 percent exhaust through a charcoal filter under yellow lights.

RESULTS AND DISCUSSION

INITIAL ASSAY OF MUNITIONS PRESENT IN REPROCESS SOLVENT FOR MUTAGENIC ACTIVITY

To determine whether the munitions (and their known degradation products) were responsible for the mutagenic activity noted in the reprocess solvent, RDX, MHX, TAX and SEX were tested in the Ames Bacterial Mutagenic Assay using the four strains of Salmonella typhimurium: TA98, TA100, TA1537 and TA1538. Results are shown in Tables 1, 2, 3, and 4, respectively with and without the presence of metabolic activation fraction S-9. The plate incorporation assay was used. Control carcinogens benzo(a)pyrene and 2-aminofluorene indicate that the S-9 preparation purchased from Litton was active. The background stippling was normal for all plates indicating that the compounds were not toxic at the concentrations used. A high of 1.0 mg/plate in 0.1 ml was used in accordance with the report received from Letterman Army Institute of Research on the mixtures of DMSO samples. The results indicate that none of the four products is apparently mutagenic in the strain of Salmonella TA98 and TA100. A slight positive response was observed using strain TA1537 (Table 3) but this could not be confirmed with a subsequent assay (Table 5). RDX also yielded positive results in tester strain TA1538 (Table 4) and this was confirmed in later assays (Table 6). A positive assay is 2x the background or a positive statistically significant slope.

TABLE 1. MUTAGENICITY OF MUNITIONS BREAKDOWN PRODUCTS IN SALMONELLA TA98.

		Concentration (mg/0.1 ml)			
Compound	S9	0.01	0.05	0.2	1.0
RDX	+	20[a]	21	20	26
	-	24	24	29	23
HMX	+	28	24	20	26
	-	25	23	27	21
TAX	+	21	65	19	19
	-	22	31	16	25
SEX	+	40	40	37	41
	-	28	30	29	33
DMSO	+	59			
	-	43			
buffer	+	69			
	-	36			
9AA (50 µg)	+	44			
	-	24			
B(a)P (10 µg)	+	96			
	-	36			
2AF (50 µg)	+	771			
	-	224			

[a] Revertants per plate. Background stippling of small colonies appeared normal at all concentrations of agents.

TABLE 2. MUTAGENICITY OF MUNITIONS BREAKDOWN PRODUCTS IN SALMONELLA TA100.

Compound	S9	Concentration (mg/0.1 ml) 0.01	0.05	0.2	1.0
RDX	+	42[a]	36	45	43
	-	58	50	47	52
HMX	+	42	45	46	48
	-	47	53	58	63
TAX	+	45	44	46	46
	-	51	49	53	50
SEX	+	70	71	50	49
	-	64	60	48	54
DMSO	+	31			
	-	42			
buffer	+	51			
	-	35			
9AA (50 ug)	+	28			
	-	29			
B(a)P (10 ug)	+	108			
	-	43			
2AF (50 ug)	+	208			
	-	65			

[a]Revertants per plate. Background stippling of small colonies appeared normal at all concentrations of agents.

TABLE 3. MUTAGENICITY OF MUNITIONS BREAKDOWN PRODUCTS IN SALMONELLA TA1537.

Compound	S9	Concentration (mg/0.1 ml) 0.01	0.05	0.2	1.0
RDX	+	19[a]	24	13	29
	-	20	23	10	34
HMX	+	8	9	6	8
	-	10	11	9	9
TAX	+	20	18	22	21
	-	23	21	17	12
SEX	+	10	5	6	7
	-	6	8	9	11
DMSO	+	8			
	-	14			
buffer	+	5			
	-	11			
9AA (50 µg)	+	251			
	-	456			
B(a)P (10 µg)	+	64			
	-	20			
2AF (50 µg)	+	31			
	-	15			

[a]Revertants per plate. Background stippling of small colonies appeared normal at all concentrations of agents.

TABLE 4. MUTAGENICITY OF MUNITIONS BREAKDOWN PRODUCTS IN SALMONELLA TA1538.

Compound	S9	Concentration (mg/0.1 ml) 0.01	0.05	0.2	1.0
RDX	+	21[a]	33	61	158
	-	11	21	43	128
HMX	+	28	28	24	19
	-	19	14	14	12
TAX	+	23	30	21	26
	-	12	16	14	11
SEX	+	40	42	40	38
	-	23	28	19	24
DMSO	+	23			
	-	9			
buffer	+	21			
	-	14			
9AA (50 µg)	+	15			
	-	8			
B(a)P (10 µg)	+	111			
	-	13			
2AF (50 µg)	+	1146			
	-	154			

[a]Revertants per plate. Background stippling of small colonies appeared normal at all concentrations of agents.

TABLE 5. MUTAGENICITY OF RDX IN SALMONELLA TA1537.

Compound	S9	Concentration (mg/0.1 ml) 0.01	0.05	0.2	1.0
RDX	+	15[a]	21	7	16
	-	16	10	14	10
DMSO	+	19			
	-	25			
buffer	+	20			
	-	19			
9AA (50 µg)	+	222			
	-	337			
B(a)P (10 µg)	+	222			
	-	337			
2AF (50 µg)	+	50			
	-	27			

[a]Revertants per plate. Background stippling of small colonies appeared normal at all concentrations of agents.

TABLE 6. MUTAGENICITY OF RDX IN SALMONELLA TA1538.

Compound	S9	Concentration (mg/0.1 ml) 0.01	0.05	0.2	1.0	1.5
RDX	+	16[a]	34	58	188	109
	-	12	16	40	90	71
DMSO	+	12				
	-	10				
buffer	+	10				
	-	12				
B(a)P (10 µg)	+	21				
	-	6				
2AF (50 µg)	+	1184				
	-	292				

[a]Revertants per plate. Background stippling of small colonies appeared normal at all concentrations of agents.

INITIAL FRACTIONATION AND TESTING OF EVAPORATOR REPROCESS SOLVENT FOR MUTAGENIC ACTIVITY

The initial findings that RDX yielded a positive result when tested in Salmonella TA 1537 suggested that this munitions component could be responsible for the mutagenic activity of the evaporator reprocess solvent. However, the biologic activity of RDX was noted only at high concentrations (1.0 mg/0.1 ml). Therefore, a HPLC method for separating and quantitating the major munitions components was developed and the concentrations of each were assayed in evaporator reprocess solvent. As shown in Figure 1, SEX, HMX, TAX and RDX were easily separated on a C_{18} column (Varian 1.2 x 25 cm). The solvent composition was 30% methanol/70% water for the first 15 min. This was programmed linearly to 100% methanol at 30 min. Solvent flow rate was 2.5 ml/min and compounds were detected at 254 nm. Table 7 lists the retention times, UV maxima and concentrations of each of the major munitions components in the sample of evaporator reprocess solvent. The observed concentrations of RDX in the evaporator reprocess solvent (0.36 mg/ml) were substantially lower than that used in the tests to determine whether the mutagenic activity in the reprocess solvent was due to any of the known munitions components. Further mutagenicity testing of RDX in TA1537 yielded variable results; no differences were observed between standard RDX prepared in DMSO and RDX which had been purified by preparative HPLC (Table 8). Moreover, the increase in revertants over solvent control was not dependent upon the amount of RDX added. Additional testing of the biologic activity of RDX in the Sister Chromatid Exchange Assay (see Table 17 in the Sister Chromatid Exchange section of this report) indicated the material was inactive.

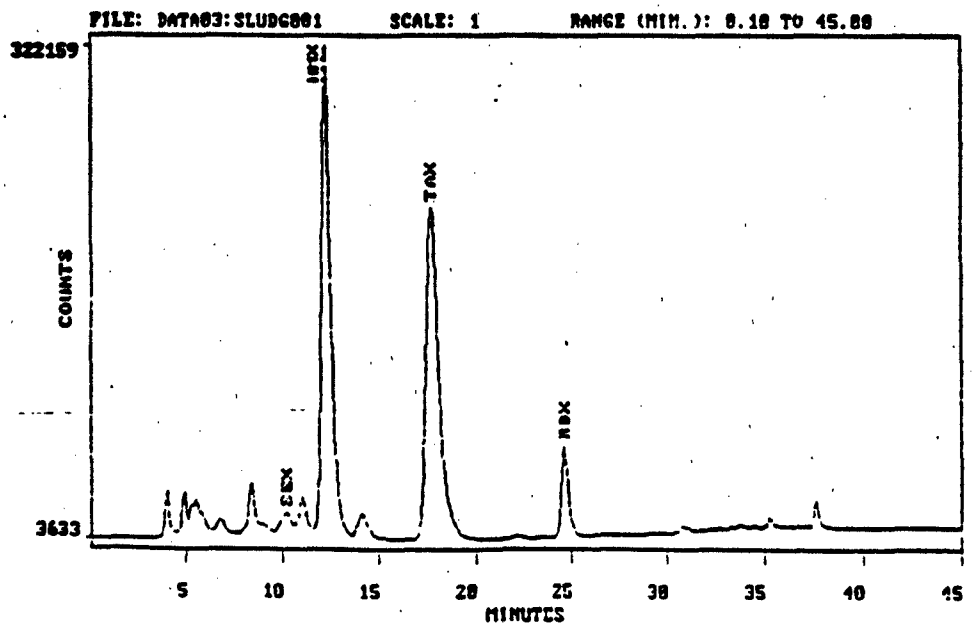


Figure 1. Chromatographic profile showing the elution of known munitions components of evaporator sludge.

TABLE 7. CONCENTRATIONS OF MUNITIONS COMPONENTS IN EVAPORATOR SLUDGE DETERMINED BY HPLC.

Compound	Retention Time (min)	Concentration (mg/ml)	UV Maximum (nm)
SEX	10	0.14	236
HMX	12	2.00	228
TAX	17	2.15	236
RDX	24	0.36	233-236

TABLE 8. MUTAGENICITY OF THE MUNITIONS COMPONENTS OF EVAPORATOR SLUDGE IN TESTER STRAIN TA1537

Compound	Concentration (mg/ml)	µl added	Revertants/Plate (Mean ± S.E.)
	Experiment I		
Evaporator Sludge		50	17±3
		100	17±2
		200	17±2
RDX	0.36	50	17±5
		100	16±4
		200	15±6
		300	5±3
9AA	2.0	25	40±17
	4.0	25	900±80
DMSO		100	6±3
Spontaneous Revertants			5±1
	Experiment II		
Evaporator Sludge		50	15±4
		100	26±5
		200	31±7
RDX	0.36	50	6±4
RDX (Purified)	0.36	50	10±4
		100	12±5
		200	23±8
SEX	2.15	50	6±3
		100	5±4
		200	5±1
Control (DMSO)		100	14±3
Spontaneous Revertants			11±5
9AA	2.0	50	84±14

TABLE 9. MUTAGENICITY OF THE PRECIPITATE AND SUPERNATANT FROM REPROCESS SOLVENT SAMPLES EVAPORATED AND RECONSTITUTED IN 30% METHANOL/70% WATER.

Sample	Revertants/Plate (TA1538)			
	+ S-9		- S-9	
	Run No. 1	Run No. 2	Run No. 1	Run No. 2
Spontaneous Revertants	26	8	13	11
DMSO	19	9	9	7
Reprocess Solvent	104	44	51	48
Dried Reprocess Solvent Reconstituted in Methanol/Water	150	93	87	80
Precipitate	13	27	7	21
Benzo(a)pyrene (10 µg)	134	63	28	12
2-Aminofluorene (50 µg)	309	566	173	173
2-Anthramine (2.5 µg)	228	431	12	10

Early attempts to isolate the mutagenic component(s) of DMSO reprocess solvent showed that there was a significant loss in mutagenic activity during the process of sample preparation and high pressure liquid chromatography. In addition, the high lipophilic action of DMSO makes this solvent unsuitable as an injection solvent for HPLC when used in large volumes. Since preparative isolation of the mutagen(s) would be necessary for structural identification, methods were explored for removing the solvent. Simple evaporation of the solvent under N_2 was time consuming and resulted in significant losses of activity in the Ames tester strain TA1537. Rotary evaporation at room temperature yielded an oily residue, when dissolved in methanol and brought to 70% water/30% methanol formed a white, crystalline precipitate. The precipitate and supernatant were tested in the Ames assay and all of the detectable activity was found in the supernatant (Table 9). All subsequent procedures employed this concentration step-rotary evaporation to remove DMSO followed by reconstitution in methanol and addition of water to bring the solution to 70% water. The supernatant removed in this step was used in all subsequent chromatography.

Standard curves were prepared with authentic samples of SEX, HMX, TAX and RDX in DMSO. Linear responses (correlation coefficients > 0.9999) of peak area vs amount of munition compound injected was observed. Concentrations of each of the components were obtained by chromatographing 10 µl of evaporator reprocess solvent by HPLC.

Evaporator reprocess solvent was rotary evaporated, reconstituted in 30% methanol/water and chromatographed on a semipreparative C18 column. Column eluate was divided into four fractions (0-11.5 min, 11.5-17 min, 17-28 min and 28 to 45 min) and each fraction of the column eluate was evaporated to dryness under vacuum, reconstituted in DMSO and submitted for both Ames assay and for tests in the Sister Chromatid Exchange Assay. The results, shown in Table 10 were repeated on several occasions with numerous different samples. Mutagenic activity was observed in the late eluting fraction (28-45 min). However, it should be noted that these results were variable and that the number of revertants per plate in collected fraction 4 was not always > 2.5 times the background. As the data in Table 10 indicate, metabolic activation was not necessary for the mutagenic activity.

TABLE 10. MUTAGENICITY OF EVAPORATOR PROCESS SOLVENT AND CHROMATOGRAPHIC FRACTIONS DERIVED FROM SEPARATION OF COMPOUNDS FROM THE PROCESS SOLVENT.

Test Article	Amount added µl	Revertants/Plate (Mean ± S.D.) (TA1538) +S9	-S9
Evaporator Reprocess Solvent	50	9±3	16±8
	100	23±5	23±7
	200	24±16	40±10
Fraction 1		9±2	8±3
Fraction 2		6±2	7±1
Fraction 3		6±4	8±2
Fraction 4		17±3	20±5
DMSO Solvent		4±2	8±2
Spontaneous revertants		4±2	12±2
2-Anthramine		129±45	232±41

RESPONSE OF AMES TESTER STRAINS TA97, 98, 100, 1535, 1537 AND 1538 AND CONFIRMATION OF MUTAGENIC ACTIVITY IN HPLC FRACTION 4

Because the Ames assay results of fraction 4 were variable and responses with evaporator reprocess solvent were at or near the levels of detectability, additional chromatographic partitioning of the reprocess solvent was performed and the fractions and evaporator reprocess solvent were tested in all of the Ames tester strains available. The results shown in Table 11 indicate that strain TA97 both with and without metabolic activation showed approximately double the number of revertants compared to solvent control using 100 µl of evaporator reprocess solvent and there was a reasonable dose response for 50, 75 and 100 µl of sample. Tester strain TA 98 appeared to be more sensitive than TA97. The number of revertants/plate was 4 times control at 100 µl evaporator reprocess solvent and again, there was a relationship between dose and response. TA98 with metabolic activation appeared to be more sensitive to the mutagen(s) in evaporator reprocess solvent than without S9 fraction. Tester strain TA100 appeared to be insensitive to the mutagenic component of evaporator reprocess solvent. Even at 100 µl, the number of revertants/plate both with and without metabolic activation was less than twice the solvent control. Similarly, tester strain TA1535 appeared less sensitive than TA98 in that the number of revertants/plate was less than twice the solvent control using 100 µl of sludge sample. Strains TA1537 and 1538 both showed positive, dose-dependent responses with evaporator reprocess solvent in the presence and absence of S9 fraction. These studies indicated that use of TA97, 1537, or 1538 was possible and that detecting the mutagen would be difficult based on its weak responses. Therefore, these tester strains would likely yield adequate sensitivity when used in combination with one of the mammalian test systems to detect and identify the mutagenic component in the samples fractionated by HPLC.

In addition to determining which of the Ames tester strains was most sensitive to unfractionated evaporator reprocess solvent, further experiments were performed to assess the activity of the tester strains with the HPLC fractions. The data in Table 12 indicates that tester strain TA1537 yielded the best response to both unfractionated evaporator reprocess solvent and to HPLC fraction 4. To examine the reproducibility of our findings, mutagenic activity using tester strain TA1537 was assessed in four separate experiments with evaporator reprocess solvent and with all four HPLC fractions. Mutagenic activity was consistently observed in HPLC fraction 4 (23-45 minutes, see Figure 2 for a typical chromatographic profile) (Table 13). These experiments confirmed the previous findings showing a weak, concentration dependent response of evaporator reprocess solvent and the presence of activity only in fraction 4. In sum, the studies conducted to this point suggested that the compound(s) was a relatively weak, direct acting mutagen which was relatively nonpolar (based on its retention on C_{18} columns).

TABLE 11. EVALUATION OF SALMONELLA TESTER STRAINS TA97, 98, 100, 1537 AND 1538 FOR SENSITIVITY TO POSITIVE CONTROLS AND TO EVAPORATOR REPROCESS SOLVENT SAMPLES.

Tester Strain	Sample		Revertants/Plate (Mean ± S.D.) + S-9	- S-9
TA97	Spontaneous Revertants		141±18	108±24
	DMSO (solvent control)		135±7	79±18
	Evap. Reproc. Solv.	50 µl	190±31	213±30
		75 µl	241±22	224±23
		100 µl	268±14	266±15
	2AA (2.5 µg)		483±117	134±44
	9AA (5 µg)		-	22±13
TA98	Spontaneous Revertants		22±7	30±8
	DMSO (solvent control)		24±4	14±4
	Evap. Reproc. Solv.	25 µl	50±6	41±5
		50 µl	71±5	41±15
		100 µl	88±17	40±10
		150 µl	111±10	56±6
	2AA (2.5 µg)		1761±271	27±10
	9AA (5 µg)		-	773±21
TA100	Spontaneous Revertants		111±18	111±16
	DMSO (solvent control)		102±2	101±12
	Evap. Reproc. Solv.	50 µl	190±31	213±16
		100 µl	268±14	266±13
	2AA (2.5 µg)		483±117	134±9
	Na Azide (5 µg)		-	1022±78
TA1535	Spontaneous Revertants		19±9	21±2
	DMSO (solvent control)		26±4	20±3
	Evap. Reproc. Solv.	50 µl	26±5	31±5
		100 µl	39±8	30±6
	2AA (2.5 µg)		199±24	23±4
	Na Azide (5 µg)		-	756±35
TA1537	Spontaneous Revertants		7±4	8±4
	DMSO (solvent control)		9±2	7±3
	Evap. Reproc. Solv.	25 µl	12±1	7±3
		50 µl	21±6	17±4
		100 µl	37±5	18±4
	2AA (2.5 µg)		164±30	9±2
	9AA (5 µg)		-	64±10
TA1538	Spontaneous Revertants		21±7	18±2
	DMSO (solvent control)		20±3	18±2
	Evap. Reproc. Solv.	50 µl	45±6	46±5
		75 µ	88±8	103±9
		100 µl	90±8	90±16
	2AA (2.5 µg)		1544±113	23±5
	9AA (5 µg)		-	703±101

TABLE 12. MUTAGENICITY OF EVAPORATOR SLUDGE AND EVAPORATOR SLUDGE SAMPLES FRACTIONATED BY HPLC IN TESTER STRAINS TA97, 98, 1537 AND 1538.

Tester Strain	Sample	Revertants/Plate (Mean ± S.D.) + S-9	- S-9
TA97	Spontaneous Revertants	151±14	172±16
	DMSO (100 µl)	141±8	170±25
	2AA (2.5 µg)	1352±149	168±24
	2NF (10 µg)	-	329±40
	Evap. Reproc. Solv. (100 µl)	237±15	278±19
	HPLC Fraction No. 1	169±24	175±9
	" Fraction No. 2	135±7	172±17
	" Fraction No. 3	158±19	164±27
	" Fraction No. 4	206±19	249±8
TA98	Spontaneous Revertants	18±5	19±5
	DMSO (100 µl)	22±6	22±3
	2AA (2.5 µg)	1571±264	17±3
	2NF (10 µg)	-	421±37
	Evap. Reproc. Solv. (25 µl)	40±9	25±7
	HPLC Fraction No. 1	36±3	25±10
	" Fraction No. 2	20±7	24±7
	" Fraction No. 3	-	21(n=2)
	" Fraction No. 4	32±4	23±2
TA1537	Spontaneous Revertants	5±4	15±2
	DMSO (100 µl)	8±2	22±8
	2AA (2.5 µg)	172±13	13±4
	9AA (50 µg)	-	94±33
	Evap. Reproc. Solv. (100 µl)	56±5	18±6
	HPLC Fraction No. 1	37±9	24±2
	" Fraction No. 2	-	-
	" Fraction No. 3	8±2	21±4
	" Fraction No. 4	28±10	23±8
TA1538	Spontaneous Revertants	14±4	10±1
	DMSO (100 µl)	9±3	8±1
	2AA (2.5 µg)	953±256	9±4
	2NF (10 µg)	-	733±48
	Evap. Reproc. Solv. (25 µl)	27±4	21±6
	HPLC Fraction No. 1 (25 µl)	13±3	8±1
	" Fraction No. 2	13±1	7±0
	" Fraction No. 3	12±5	10±4
	" Fraction No. 4	23±9	19±1

TABLE 13. REPETITIVE ASSAYS OF EVAPORATOR SLUDGE AND HPLC FRACTIONS PREPARED FROM EVAPORATOR SLUDGE FOR MUTAGENIC ACTIVITY WITH TA1537.

Sample	Revertants/Plate (Mean ± S.D.) Trial 1	Trial 2	Trial 3	Trial 4	Mean (-Solv.)
			With S9		
DMSO	7±4	5±3	6±3	8±4	--
ERS* 50	8±3	24±5	18±2	19±2	11
ERS 100	44±5	32±4	39±9	38±11	32
ERS 200	75±11	55±3	60±15	63±8	57
Fract 1	---	12±2	---	---	7
Fract 2	9±2	9±4	---	---	3
Fract 3	16±7	7±3	---	---	5
Fract 4	48±3	44±9	44±8	30±6	35
			Without S9		
DMSO	20±7	15±6	15±5	15±4	--
ERS 50	---	31±4	22±6	34±8	13
ERS 100	53±5	42±10	33±6	41±5	26
ERS 200	85±10	58±6	54±5	59±17	48
Fract 1	---	16±2	---	---	1
Fract 2	24±5	24±6	---	---	6
Fract 3	27±3	26±6	---	---	9
Fract 4	52±12	48±6	53±5	33±9	30

* ERS = Evaporator Reprocess Solvent

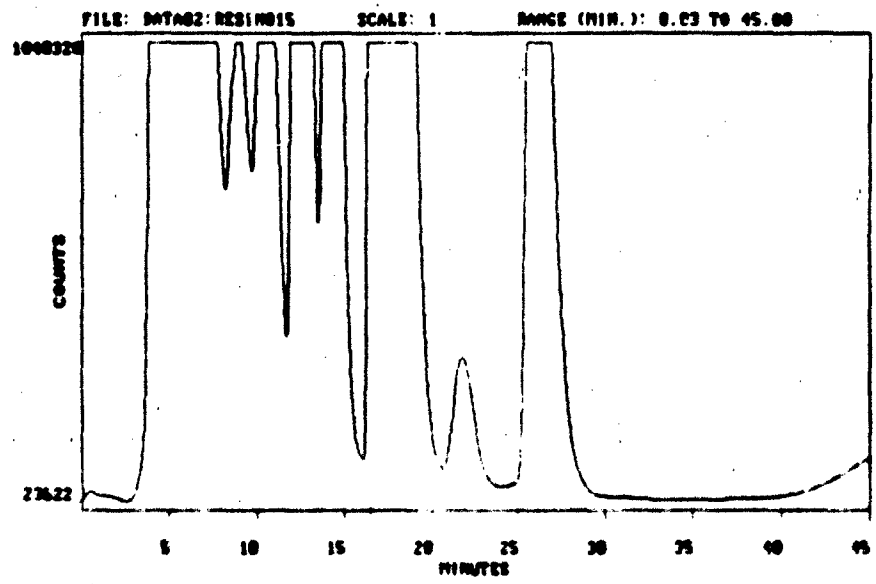


Figure 2. UV chromatographic profile (UV at 254) of 10 ml evaporator reprocess solvent sample evaporated to dryness under vacuum and reconstituted in 2 ml methanol/water (30:70). The entire sample was injected onto a C18 semi-preparative column and eluted with 30% methanol/70% water for 15 min followed by linear programming to 100% methanol over 15 min. The solvent flow rate was 2.5 ml/min and samples of column elute were collected from 0-12.5 min, 12.5-16 min, 16-22.5 min and 22.5-45 min. Each sample was evaporated to dryness under vacuum, reconstituted in DMSO, and submitted for Ames assay.

ATTEMPTS TO PURIFY THE MUTAGENIC COMPONENT(S) OF EVAPORATOR SLUDGE

Based on the small quantitites of compound likely present as a mutagenic contaminant in evaporator reprocess solvent, mass spectrometry offered the best possibility of being able to identify the component. Introduction of the sample by direct probe insertion would offer the advantage of being able to test a fraction of the sample using the bioassay. However, the disadvantage is that the sample must be pure before interpretable spectra can be obtained. Alternative approaches were gas chromatography/mass spectrometry with fraction 4 and to attempt to identify all of the compounds present in the sample. Once identified, authentic compounds could be purchased or prepared synthetically for testing in the Ames assay. Putative assignment of the compound responsible for the mutagenicity would depend upon a demonstration of mutagenic activity in sensitive Ames strains at the levels of compound found in evaporator reprocess solvent.

Several attempts were made to subfractionate the material eluting from the HPLC column between 28.5 and 45 min and to obtain discrete sections that were relatively pure. Initial trials involved collecting fractions at 4-5 minute intervals over the period of time (28-45 min) in the elution of evaporator

reprocess solvent known to contain the mutagenic activity. Thus, fractions of the column eluate were collected at 28.5-32, 32-36, 36-40 and 40-45 min and the fractions were evaporated. The residue was reconstituted in DMSO and submitted for Ames assays. Significant activity above background was obtained in evaporator reprocess solvent but not in any of the collected HPLC fractions (Table 14). These fractions were retested using tester strain TA1538 and there appeared to be some activity in the 28-32 min and 32-36 min collections. Additional quantitites of these two fractions were prepared and submitted for mass spectrometry.

TABLE 14. MUTAGENICITY OF SUBFRACTIONS COLLECTED FROM THE HPLC COLUMN IN THE AREA OF THE CHROMATOGRAM PREVIOUSLY SHOWN TO CONTAIN MUTAGENIC ACTIVITY.

Test Substance	µl Used	Revertants/Plate (Mean ± S.D.) TA1537	TA1538
Fraction 4A (28.5-32 min)	50	14±5	--
	100	15±2	--
	200	20±6	60
Fraction 4B (32-36 min)	50	20±3	--
	100	21±6	--
	200	23±3	60
Fraction 4C (36-40 min)	50	19±5	--
	100	17±4	--
	200	12±2	30
Fraction 4D (40-45 min)	50	15±3	--
	100	17±4	--
	200	17±7	30
Evaporator Reprocess Solvent	50	28±3	71±6
	100	41±4	128±12
	200	64±8	213±14
DMSO		15±6	16±7
Spontaneous Revertants		18±4	18±6
9AA		51±5	--
2NF			1033±84

MASS SPECTROMETRY OF SAMPLES COLLECTED FROM 28.5-32 and 32-36 MIN

The samples collected from 28.5 to 32 min and from 32 to 36 min were evaporated and reconstituted in methanol. The final sample was dried on a glass probe and mass spectra were obtained on a VG ZAB2F high resolution mass spectrometer in chemical ionization mode. Numerous fragments were observed, none of which were clearly identifiable, thereby indicating that additional purification steps would be necessary before interpretable spectra could be obtained.

ATTEMPTS TO PURIFY AND IDENTIFY THE MUTAGEN USING AN ISOCRATIC SOLVENT

Some of the potential contaminants in fraction 4 may be derived from concentration of organic contaminents in the water mobile phase which are subsequently eluted during the relatively high methanol concentrations that occur during the terminal phases of the HPLC run. In an effort to further fractionate fraction 4 and to obtain a sample sufficiently pure for mass spectral analysis, chromatography of the residue derived from fraction 4 was attempted isocratically. Samples were prepared by evaporating the evaporator reprocess solvent, reconstituting the residue in 30% water/70% methanol followed by injection onto a C18 column. Fraction 4 was collected as described earlier, the solvent was removed under vacuum and the sample was reconstituted in methanol/water (70/30). The sample was then chromatographed on the same C18 column using an isocratic mobile phase of 80% methanol/20% water. As indicated in Figure 3, numerous UV absorbing components of the residue were separated by this chromatographic step. Fractions of the column eluate were collected (0-5.5, 5.5-8, 8-11 and 11-17 min), the solvent was evaporated and the residue was dissolved in DMSO for Ames assay (Table 15). These studies showed that all four fractions contained material that tested positive in the Ames assay. Based on these findings and our apparent inability of provide a discrete HPLC fraction that contained clearly identifiable biologically active material, we concluded that attempts to identify all components of "fraction 4" followed by biologic testing of suspect compounds might yield the best approach to the identification of the mutagen(s) in evaporator reprocess solvent. The appendix of this report contains some of the extensive GC/MS analysis of "fraction 4" done by Dr. Brian Andresen at Lawrence Livermore National Laboratory (Livermore, CA).

THE RECRYSTALLIZATION SOLVENT'S "FRACTION 4" WAS ASSAYED BY GC-MS

The elute assayed was from a semiprep C_{18} column between 28 and 36 minutes using a motile phase of 30% methanol/705% water isocratically for 15 minutes followed by a linear gradient to 100% methanol over 15 minutes.

Analysis of the evaporated HPLC fractions was done by gas chromatography/mass spectrometry using electron impact ionization. Both total ion current and selected ions (m/z = 46, indicative of the NO_2 functionality) were monitored. Portions of each fraction were derivatized with silylating reagent to prepare volatile trimethylsilane (TMS) derivatives of alcohols, phenols, carboxylic acids and amines. All chromatography was performed on a 30 m DB-1 column (J and W Scientific, Rancho Cordova, CA). The initial oven temperature was 70°C and this was programmed linearly to 300°C at 10°/min.

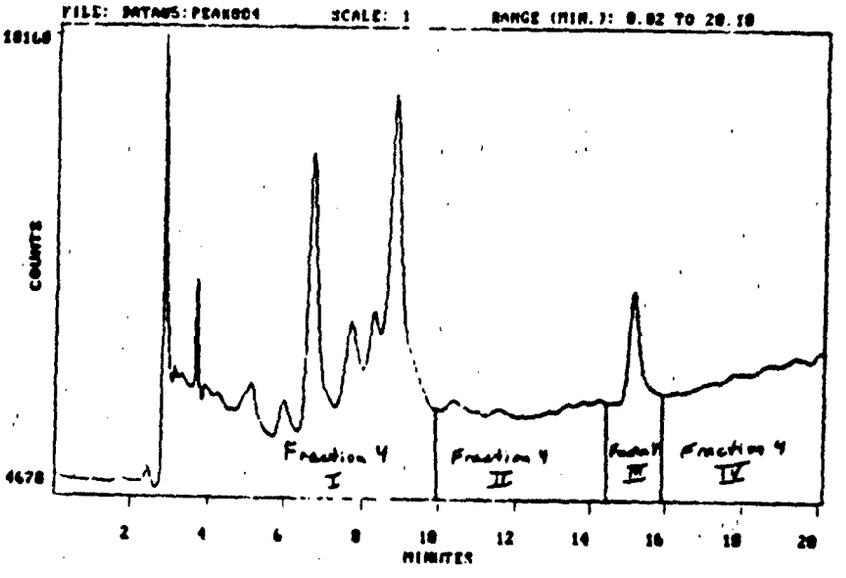


Figure 3. Chromatographic profile of UV absorbing components eluting from a C18 reversed phase column run isocratically at 80% methanol/20% water. Evaporator sludge was chromatographed using a linear gradient of water to methanol and fraction 4 containing the major portion of the mutagenic components of the sample was evaporated and reconstituted in 70% methanol/water. This sample was injected onto the C18 column and eluted isocratically with 80% methanol/water. Fractions of the column eluate were collected as indicated and submitted for Ames testing.

The results are detailed in the appendix. Both underivatized and derivatized samples showed the presence of numerous placticizers (butyl-benzyl-phthalate, dioctylphthalate, and dioctylacetate) which are almost certain to be due to the fact that the evaporator sludge was shipped and stored in plastic containers. The TMS derivatives were formed from some of the plasticizers and some of the hydrocarbons. Selected ion monitoring for m/z = 46 showed a small response at 13-14 min. The quantities present were insufficient for identification.

The samples by this time had "aged" to the point where the biologic (Ames) activity had decreased beyond a level facilitating further isolation. The implications are that although plasticizers from sample handling/storage prior to our receipt may have accounted for some of the activity, the HPLC isolation of "active fraction 4" suggests that other process-related compounds were involved. A new "run" of the process coupled rapid careful handling and assay should resolve the issue.

TABLE 15. ACTIVITY OF HPLC SUBFRACTIONATION OF "FRACTION 4" PREPARED BY ISOCRATIC HPLC.

Test Substance	µl Tested	Revertants/Plate (Mean ± S.D.) TA1538
Fraction 1	50	15±5
(0-5.5 min)	100	24±4
	200	33±8
Fraction 2	50	11±3
(5.5-8.0 min)	100	13±4
	200	20±3
Fraction 3	50	10±2
(8.0-11.0 min)	100	14±4
	200	27±3
Fraction 4	50	20±4
(11.0-17.0 min)	100	43±4
	200	65±7
Evaporator Reprocess Solvent	50	48±3
	100	86±10
	200	127±14
DMSO		8±4
Spontaneous Revertants		7±3
2NF		911±101

MUTAGENICITY EVALUATION OF FRACTION 4 OF EVAPORATOR SLUDGE IN MOUSE LYMPHOMA CELL

Introduction

As a part of the project, a mammalian cell mutagenicity assay was included to confirm the mutagenic activity detected by Ames bacterial mutagenicity assay. This report describes the mutagenic results that were obtained by the mouse lymphoma cell (L5178Y) mutagenicity assay. Fraction 4 of the evaporator sludge, separated by high performance liquid chromatography (HPLC), was tested at three concentrations of the original evaporator sludge, 2, 4, and 8% v/v. The evaporator sludge, 2 and 4% v/v, and known mutagen, ethyl methanesulfonate (EMS) were included as control standards. The mouse lymphoma mutagenicity assay was conducted under standard operating procedure without the addition of S-9 activation.

The results indicate that the HPLC fraction 4 that was separated from the evaporator sludge containing direct-acting mutagen by mouse lymphoma assay, which was also positive in the Ames mutagenicity assay, lacked mutagenic activity in mouse lymphoma cells when tested at comparable equivalent concentrations of the mutagen(s) containing evaporative sludge.

The possible reasons for the differences are discussed.

Materials and Methods

Test Sample: Fraction 4 of Evaporator Sludge

Fraction 4 of evaporator sludge which was fractionated by high performance liquid chromatography (HPLC) and concentrated 20-fold of the original evaporator sludge was received from Dr. Buckpitt. The sample which was solubilized in 100% DMSO was diluted in tissue culture media and evaluated by exposing mouse lymphoma cells for 4 hours at 37°C. In addition to the fractionated sample, we received an aliquot of the original evaporator sludge which was tested concurrently. The known mutagen ethyl methanesulfonate (EMS) which was purchased from Sigma Chemical Co. was used as a standard direct-acting mutagen.

Mouse Lymphoma Mutagenicity Assay

The mouse lymphoma mutagenicity assay was conducted as previously described by Clive and Spector (1975) with modification set forth in the standard operating procedure (SOP). The modification involved the method in which cells were placed onto the agar. In the modification, aliquots of treted (exposed) cells were dispensed independently into 3 or 4 replicate dishes of cloning agar medium following the thrid passage of the cells. The mouse lymphoma cells L5178Y (subclone 3.7.2C) were received from Dr. D. Clive (Burroughs Wellcome Co.). Briefly, the cells were exposed to the test sample for 4 hours and then the mutated cells were given the opportunity for expression of the mutation by daily passaging the cells in fresh media for three days. After the third passage the cells (1×10^6 cells) were cloned in soft agar with and without trifluorothymidine (TFT). After the clonal growth was determined, the mutation rate was expressed as mutants/1×10^6 cells.

Results and Discussion

The test sample, fraction 4 of the evaporator sludge, was tested at 3 concentrations, 2, 4, and 8% v/v, without S-9 activation on actively growing mouse lymphoma cells after 4 hour exposure. The control samples included the original unfractionated evaporator sludge and known direct-acting mutagen. The results are summarized on Table 16. Although fraction 4 was tested at 2-fold higher concentration than the unfractionated evaporator sludge which was mutagenic at 4% v/v, the fractionated sample lacked mutagenic activity while mutagenic activity was detected in the unfractionated sample. Since the original evaporator sludge and control mutagen were positive, the assay was functional and the results real. The reason for absence of mutagenic activity in mammalian cells is not known since the same fraction was mutagenic in the bacterial cells. Although the specific reason for the differences is not known, it is possible that either the specific bacterial strain used is more sensitive than the mouse lymphoma cells for detection of mutagens or the unfractionated evaporator sludge contains several weak mutagens which when separated are undetectable by the mouse lymphoma assay. Further studies on the chemically defined mutagen(s) in the evaporator sludge are needed to explain the differences between the two assays.

TABLE 16. EVALUATION OF FRACTION 4 FOR MUTAGENIC ACTIVITY IN MOUSE LYMPHOMA CELLS

Sample	Dose	% Cloning Efficiency	Mutants/10^6 Mean ± SD
Control	2% DMSO	100	20.5 ± 4.4
Control	4% v/v DMSO	100	25.5 ± 5.3
Fraction 4	2% v/v *	100	26.3 ± 2.9
Fraction 4	4% v/v	100	24.3 ± 5.3
Fraction 4	8% v/v	100	21.8 ± 6.1
Evap Sludge	2% v/v	89	24.8 ± 5.1
Evap Sludge	4% v/v	100	69.0 ± 4.7
EMS**	2.5 mM	100	195.0 ± 11.4

*Concentration equivalent to unfractionated evaporator sludge.

**Control mutagen - ethyl methanesulfonate.

SISTER CHROMATID EXCHANGE

Introduction

The results of in vitro mutagenic assays are rendered more valuable with the inclusion of additional mutagenic/clastogenic tests. The sister chromatid exchange (SCE) assay is a sensitive method of determining the mammalian cell DNA-damaging ability of a test agent; damage is exhibited via exchanges between sister chromatids (Stetka and Wolff, 1976). The ability of a compound to produce an increase in SCE over the baseline value (usually 7-9 SCE/cell) has been correlated to the mutagenicity of that compound (Wolff, 1977).

SCEs are visible through the light microscope because the sister chromatids are stained differentially. Bromodeoxyuridine (BuDR) is incorporated into the chromosomes of Chinese hamster ovary (CHO) cells for two cell divisions. Because DNA replicates semi-conservatively, after the two cell cycles one sister chromatid will be unifilarly substituted with BuDR whereas the other will be bifilarly substituted. The unifilarly-incorporated chromatid stains darkly whereas its bifilarly-incorporated sister chromatid stains lightly (Wolff, 1977).

Materials and Methods

The methods used were patterned after those of Wolff (1981). A population of 10^6 CHO cells (obtained from S. Wolff, UCSF) was seeded into each 75 cm^2 plastic tissue culture flask. Cells were cultured in McCoy's 5A medium (Gibco) supplemented with 1% glutamine and 10% fetal bovine serum, and were incubated at 37°C (5% CO_2) for 24 hours before treatment was initiated. The experiment can be divided into 3 groups according to treatment plan. Cells were exposed in one of three ways; 1 hour of treatment followed by 27 hours of recovery (Group I), 1 hour of treatment in the presence of metabolic activation, followed by 27 hours of recovery (Group II), or 28 hours of continuous exposure (Group III). The cells from Groups I and II were washed with phosphate buffered saline (PBS) solution 1 hour after exposure, then given fresh medium containing 10μM BuDR (Sigma). Group III cells were not washed after exposure; 10μM BuDR was added to the medium 1 hour after treatment. After the addition of BuDR, cells were handled only under yellow light. After 24 hours of incubation at 37°C (5% CO_2), 2 X 10^{-7}M colcemid (colchicine) was added to the medium in order to arrest the cells at metaphase. Incubation was continued for 3 hours, after which the cells were harvested by shake-off. Cells were then treated with a hypotonic solution of 0.075M KCl to assure proper chromosome spreading, and then fixed in methanol/acetic acid (3:1 v/v).

Fixed cells were dropped onto slides and allowed to air dry overnight. Staining was accomplished using a variation of the fluorescent-plus-Giemsa (FPG) technique of Perry and Wolff (1974). Slides were stained in Hoechst 33258 at a concentration of 5 μg/ml in M/15 Sorenson's buffer, pH 6.8. Following a 20 minute staining period in Hoechst, slides were exposed to UV light for 5 minutes, after which the stain was made permanent by immersion in 5% Giemsa (Gurr's R66, diluted in M/15 Sorenson's buffer, pH 6.8) for 5-10 minutes, as required. After mounting in Permabond, slides were scored with respect to frequency of SCE per cell.

Metabolic activation was used in Group II cells in an attempt to determine if any components in the sludge mixture acted as pro-mutagens instead of directly-acting mutagens. During treatment with metabolic activation, the exposure medium was changed to McCoy's 5A with 2.5% fetal bovine serum plus 10% S9 mix. The S9 preparation procedure is a variation of that used by Stetka and Wolff (1976); S9 mix consists of 10% S9 fraction from Aroclor-1254-induced rats (Litton), 0.008M $MgCl_2$, 0.033M KCl, 0.005M glucose 6 phosphate, 0.004M nicotine adenine diphosphate (NADP), and 0.1M Na_2HPO_4-NaH_2PO_4 (pH 7.4).

Untreated controls were run during all experiments. Due to experimental design limitations, the evaporator sludge (ES) concentrations for a particular exposure group could not all be tested at one time. Therefore, the concentrations within a given exposure group were compared to different untreated control values, depending on the date in which the concentration was tested. These negative control groups were handled exactly the same as treatment groups in all respects other than the addition of test agents. In addition, a positive control of 0.001M ethyl methanesulfonate (EMS) was included in order to check for assay effectiveness (28 hour exposure). For those experiments in which metabolic activation was used, the following control groups were substituted; a control treated with S9 mix only and a positive control consisting of 0.001M cyclophosphamide (CP) plus S9 (1 hour exposure followed by 27 hours recovery for both groups). In addition, cells were exposed to 15% DMSO to test for possible solvent effects.

The data for most exposure groups appeared to be distributed in a non-normal fashion and had rather large coefficients of variation. For these reasons a nonparametric statistical test (Mann-Whitney) was used to compare median SCE values derived from different slides. The chosen level of significance was $p \leq 0.05$.

Results

Table I shows that in Group I, a statistically significant increase in SCE occured for those cells exposed to 5% evaporator sludge and 10% evaporator sludge. A higher concentration of 15% ES was tested but results could not be counted due to mitotic delay and substantial cell death. When metabolic activation was present (Group II), only the 10% ES produced a statistically significant increase in SCE. After a 28 hour exposure (Group III), an increase in SCE is produced for those cells exposed to 1% ES and 2% ES (higher concentrations tested could not be scored due to cytotoxicity).

Three sets of untreated controls were used, each corresponding to a separate experiment date, and not according to experiment group. There is variation in the untreated control baseline values for SCE (Table 17). Because of this variation between experiments it is more difficult to compare the SCE values between exposure groups if those being compared were derived from different experiments; however, the increase in SCE seen after exposure to ES was sufficiently high to offset these small differences in baseline values. For all exposures (except the positive controls) 50 cells were scored; 25 from each of 2 slides. In every case but one, the SCE values obtained from one slide were not statistically different from the values obtained from the second slide of the same exposure group (Mann-Whitney, $p \geq 0.05$). The fact that one out of 36 slides scored was not statistically

TABLE 17. FREQUENCY OF SISTER CHROMATID EXCHANGE AFTER EXPOSURE TO EVAPORATOR SLUDGE (ES).

Test Agent	Experiment Date	S9	Time (hours)		Mean (SD) No. SCE/Cell		Median No. SCE/Cell	
			Exposure	Recovery	Treated	Untreated Control	Treated	Untreated Control
Group I								
ES 1%	5/21/85		1	27	8.78(3.54)	9.80(3.19)	8.50[a]	10.00
ES 2%	5/29/85		1	27	7.76(2.95)	7.98(2.93)	7.50[a]	7.50
ES 5%	5/21/85		1	27	17.98(9.42)	9.80(3.19)	15.50[b]	10.00
ES 10%	5/21/85		1	27	37.40(10.9)	9.80(3.19)	37.00[c]	10.00
ES 15%	5/21/85		1	27	NS	9.80(3.19)	NS	10.00
Group II								
ES 0.1%	5/01/85	+	1	27	7.16(2.61)	7.96(2.99)	7.00[a]	8.00
ES 1%	5/01/85	+	1	27	7.50(2.92)	7.96(2.99)	8.00[a]	8.00
ES 2%	5/29/85	+	1	27	8.46(3.01)	9.02(3.23)	8.00[a]	8.00
ES 5%	5/29/85	+	1	27	9.90(4.41)	9.02(3.23)	10.00[a]	8.00
ES 10%	5/01/85	+	1	27	23.00(11.5)	7.96(2.99)	22.00[b]	8.00
Group III								
ES 0.1%	5/01/85		28		8.16(3.09)	8.28(3.06)	8.00[a]	8.00
ES 1%	5/01/85		28		12.38(4.04)	8.28(3.06)	12.00[b]	8.00
ES 2%	5/29/85		28		24.12(7.18)	7.98(2.93)	23.00[c]	7.50
ES 5%	5/21/85		28		NS		NS	
ES 10%	5/01/85		28		NS		NS	
Control								
EMS 10^{-3}M	5/21/85		28		28.00(6.78)	9.80(3.19)	27.00	10.00
CP 10^{-3}M	5/01/85	+	1	27	82.00(17.7)	7.96(2.99)	80.00	8.00
CP 10^{-3}M	5/29/85	+	1	27	46.00(13.8)	9.02(3.23)	46.00	8.00
DMSO 15%	5/21/85		1	27	10.00(4.26)	9.80(3.19)	10.00	10.00

Legend: Fifty cells were scored for each exposure group, except for the positive controls (EMS and CP), in which 25 cells were scored. Those cells treated under the influence of metabolic activation were compared to untreated controls which were similarly exposed to S9 mix. EMS is ethyl methane sulfonate, CP is cyclophosphamide, and DMSO is dimethyl sulfoxide. NS means not scorable due to cytotoxicity. Median values with the same superscript (a,b,c) are not considered to be statistically different.

equivalent to its partner (median values were 7.0 and 8.0) is not beyond the realm of chance, and is acceptable statistically. The positive controls values were derived from 25 cells/dose; mean SCE values were above twice the baseline level. SCE values obtained from cells exposed to the solvent control (15% DMSO) were not significantly different from those obtained from the untreated group ($p \geq 0.05$, Table 10).

Discussion

In Figure 4 a dose-dependent increase in SCE was seen for all exposure groups tested (Groups I, II, III). A compound is said to effectively induce SCE if concentrations tested increase the SCE to equal to or greater than twice the background level (Latt et al., 1981). Those concentrations falling into the category of effective SCE induction included 10% evaporator sludge (Groups I and II) and 2% evaporator sludge (Group III).

Regression Equations

Group I	$y = 0.38 + 0.344\ X$	$r = 0.99$	$P<0.01$
II	$y = 0.64 + 0.199\ X$	$r = 0.90$	$P<0.025$
III	$y = 0.32 + 1.08\ X$	$r = 0.94$	$P>0.20$*

* Not significant due to too few degrees of freedom

Regression equations are based on $Y_i = \frac{\text{Mean SCE treated}}{\text{Mean SCE own control}}$

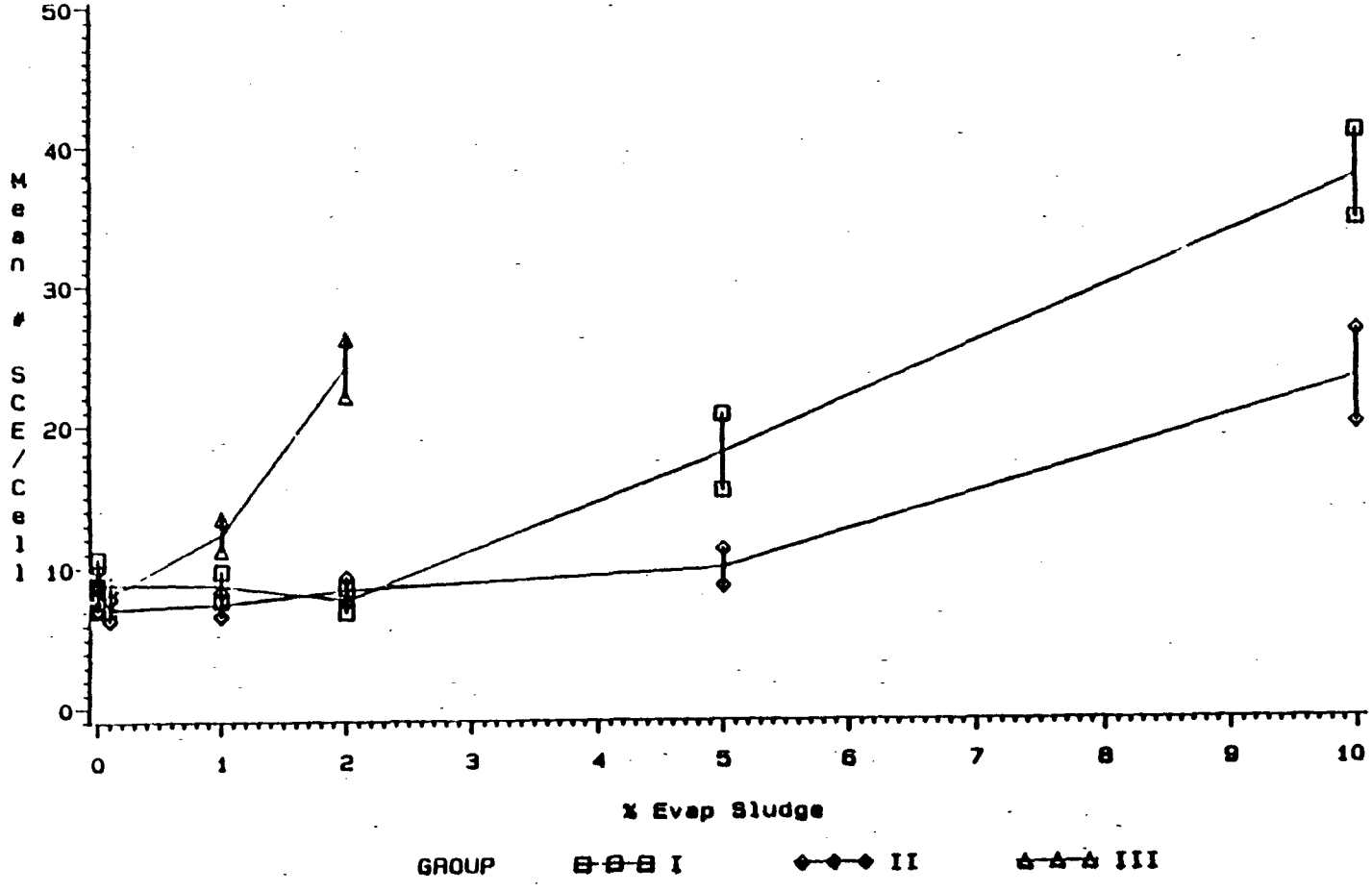
The calculation of Y_i allowed the pooling of all data in that group since there were 2 Controls/Group. Slope for Group III was 3-5 X that of Groups I and II - due to longer exposures.

No statistically significant increase in SCE above baseline was seen in those cells exposed to concentrations tested at or below the following levels: 2% evaporator sludge (Group I), 5% evaporator sludge (Group II), or 0.1% evaporator sludge (Group III). The existence of a threshold value, below which no relative increase in SCE occured, suggests either that the cells were able to exclude or eliminate small amounts of mutagen(s) or that damaged DNA was being repaired at a rate consistent with production of damage.

The presence of metabolic activation decreased the sensitivity of the assay to the effects of evaporator sludge by approximately 40-50% (as calculated by the ratio of the slopes of the dose-response lines for Groups I and II between the values of 5 and 10%); this can be expressed as No. SCE/% evaporator sludge. This decrease in activity suggests that S9 mix may have had a deactivating effect on the mutagen(s) present in evaporator sludge. In the Ames assay, the addition of S9 did not consistently decrease or increase the effectiveness of evaporator sludge's mutagenic activity (Sauers et al., 1983).

The longer exposure time (28 hour) increased the sensitivity of the test system to evaporator sludge by approximately 300% (as determined by using the method above); therefore, lower concentrations of evaporator sludge could be determined to be mutagenic. Unfortunately, effects of evaporator sludge upon mitotic delay and cell death were also enhanced by increasing exposure time.

FIGURE 4. Dose Response of SCE Relative to Dose and Exposure Group.



Legend: Mean values and 95% confidence intervals given.
Group I - 1 h exposure followed by 27 h recovery.
Group II - 1 h exposure (in the presence of metabolic activation) followed by 27 h recovery.
Group III - 28 h exposure, with no recovery period.

AMES TESTING OF "DMSO-SURROGATE"

The "Surrogate," consisting of propellant mixtures equivalent to the concentration in "Evaporator sludge" as determined by HPLC, was tested for mutagenicity using testor strains of Salmonella (Ames test) that had been employed in evaluating the original Evaporator Sludge. These concentrations of the propellants were analyzed by HPLC and confirmed the values given us by the sponsor. The "Surrogate" was not mutagenic.

The concentrations used in DMSO were:

	mg/ml
SEX	0.14
TAX	2.15
HMX	2.00
RDX	0.36

The propellant mixtures were dissolved in 100% DMSO and analyzed in 3 concentrations (50, 100 and 200 µl) per plate (Table 18).

Thus, the Salmonella strains which showed mutagenic response to the "DMSO Evaporator Sludge" did not show a mutagenic response to a surrogate consisting of a mixture of the 4 propellant compounds in the same concentrations as the sludge. The conclusion is that the mutagenic response to evaporator sludge was not due to the compounds RDX, HMX, SEX, and TAX in DMSO. Some other unidentified material(s) caused the mutagenic activity. The evaporator sludge aliquot is now so old that it no longer manifests the mutagenic activity. The active compound(s), however, persisted for several years and thus are likely fairly stable. If this munitons recrystallization process involving DMSO were to be repeated in a new pilot run, it would be prudent to determine whether mutagenic activity is present. The results of the earlier tests at this laboratory suggested that the material, "evaporator sludge," contained a potent mutagen.

The objectives of this study were achieved in that we found that the evaporator sludge in DMSO was a potent mutagen, as evidenced by both bacterial and mammalian cell tests. Also, the mutagenic activity was not due to the purported mixture in DMSO of RDX, HMX, SEX, and TAX.

TABLE 18. TEST RESULTS FOR AMES "DMSO SURROGATE."

Strain TA 100:

	Revertants/plate (Mean ± S.D.)	
Run No. 1	**-S9**	**+S9**
PBS	58 ± 6.9	100 ± 22
DMSO (100 µl)	50 ± 6.6	78 ± 8
NaA2 (5 µg)	1738 ± 79	---
2AA (2.5 µg)	---	3327 ± 147
Surrogate: 200 µl	57 ± 4.6	50 ± 5
100 µl	73 ± 8.4	73 ± 6.4
50 µl	76 ± 9.0	93 ± 17
Run No. 2	**-S9**	**+S9**
PBS	113 ± 11.5	---
DMSO (100 µl)	89 ± 9.8	---
NaA2 (5 µg)	1924 ± 71	---
Surrogate: 200 µl	77 ± 5.7	---
100 µl	97 ± 5	---
50 µl	103 ± 12.5	---

Strain TA 98:

	-S9	+S9
PBS	21 ± 2.4	25 ± 6.4
DMSO (100 µl)	17 ± 5.0	21 ± 3
2NF (5 µg)	475 ± 59	---
2AA (2.5 µg)	---	2448 ± 120
Surrogate: 200 µl	12.5 ± 2.6	18 ± 2.7
100 µl	18 ± 1.0	24 ± 4.6
50 µl	17 ± 4.4	28 ± 4

Strain TA 1537:

	-S9	+S9
PBS	8 ± 3	8 ± 2.8
DMSO (100 µl)	5 ± 1.7	5 ± 1.8
9AA	1746 ± 184	---
2AA	---	166 ± 16.3
Surrogate: 200 µl	2.9 ± 1.9	2 ± 0.9
100 µl	7 ± 2.8	6 ± 3.4
50 µl	4 ± 1.6	6 ± 2

Positive Control Concentrations

NaA2	at 5 µg/50 µl	(TA100)
2AA	at 2.5 µg/50 µl	(TA98, 100, 1537)
9AA	at 80 µg/50 µl	(TA1537)
NF	at 5 µg/50 µl	(TA98)

OVERALL DISCUSSION OF FINDINGS AND CONCLUSIONS

These studies have shown that the reprocess solvent and evaporator sludge in the DMSO munitions process contained mutagenic materials. Using reagent grade TAX, SEX, RDX, and HMX in the proportions assayed for the material as delivered, and diluted appropriately in reagent grade DMSO, the surrogate mixture showed an absence of any mutagenic activity using the technology available to us. Thus, the mutagenic activity was either the result of a contaminant or some artifact introduced as a result of the storage, or in the actual process itself with some kind of aging interactive product. Attempts using GC-MS were unsuccessful in identifying the active compound although a biodirected technique using the Ames test as an indicator of different fractions separated by HPLC is an effective technique. By the time the test were performed, the level of mutagenic activity had diminished to the point where these techniques were of inadequate sensitivity to clearly delineate which particular molecular weight species were involved. Some limited success in isolation of an active fraction was realized. As discussed on p. 26, GC-MS analyses showed the presence of plasticizers and fatty acids which may be artifacts added in post-process handling/analyses. Trace compounds were observed in the selected ion chromatograms ($m/z = 46$) which indicated the presence of components with NO_2 groups. However, due to the small quantities of these present they could not be identified chemically or tested for mutagenicity in the Ames assay.

The mouse lymphoma assay which was also positive, showed a much stronger response in the Phase I testing than in this Phase II study some three years later. The biological active, while relatively stable, apparently did lessen with time, and by about four or more years was not easily seen using the Ames assay on samples.

FINAL SUMMARY AND CONCLUSIONS

The component of the evaporator reprocess solvent that was responsible for the mutagenic activity of this munitions byproduct could not be identified. Based on the results of the Ames assay testing it is a very weak mutagen and does not require metabolic activation. Also, the Salmonella strains which showed mutagenic response to the DMSO evaporator sludge did not show a mutagenic response to a surrogate consisting of a mixture of the four propellent compounds analyzed in the same concentrations as the sludge. The mutagenic activity of the reprocess solvent could not be attributed to one of the primary munitions. The compound(s) were relatively lipophilic. The failure to provide definitive identification of the mutagen may have been due to the fact that there is more than one chemical entity responsible for the weak biologic response. Alternatively, the probable chemical instability of the mutagen, which is supported by the markedly attenuated response in the mouse lymphoma assay observed at the end of the study in comparison with that when the work was started, may have been a factor.

The first phase of this study used the reprocess mixture to confirm the presence of mutagenic activity using the Mouse Lymphoma test. A repeat of the Mouse Lymphoma test and the use of the Ames Salmonella assay as well as the use of mammalian cells to demonstrate a sister chromatid exchange effect, all pointed to the fact that compounds in this material was indeed capable of causing damage to DNA and mutation. The potential success of the study was somewhat compromised by our having to wait for more than 2 years between the time the material was generated during a pilot plant run, to when we first received samples for analysis, and by our inability to fully document the exact steps in the collection and storage of the sludge and solvent prior to delivery to our laboratory. Future pilot plant runs of this munitions process should incorporate a sampling protocol which would provide documentable fresh samples and answer questions as to the source of any potential mutagenic activity.

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DATA COLLECTION AND STORAGE

Raw data which were manually entered into hardbound laboratory notebooks and the final report are stored in Room 431, Bldg. H-215, at L.E.H.R., University of California, Davis, CA 95616. The storage area is under the control of the Quality Assurance Officer.

QUALITY ASSURANCE STATEMENT

All of the genotoxicity testing for this study was conducted following Good Laboratory Practice guidelines in a laboratory performing GLP studies.

PERSONNEL

Study Director, Principal Investigator, and Security Officer:	Marvin Goldman, Ph.D.
Co-Investigators:	Alan R. Buckpitt, Ph.D. Thomas G. Kawakami, Ph.D.
Environmental Health and Safety Officer:	Carl Foreman, B.S.
Statistician:	Leon S. Rosenblatt, Ph.D.

SIGNATURE PAGE

Final Report for Study Number 4840, APO 84PP4840, July 1988

Health Effects Research on Dimethyl Sulfoxide (DMSO) Munition Recrystallization Process Solvent, Phase II

Prepared by: Marvin Goldman
M. Goldman, Ph.D., Study Director — 1-31-89 Date

A. Buckpitt
A. Buckpitt, Ph.D., Co-Investigator — 2-28-89 Date

Thomas Kawakami
T. G. Kawakami, Ph.D., Co-Investigator — 2-28-89 Date

Reviewed by: Len S. Rosenblatt
L. S. Rosenblatt, Ph.D., Biostatistician — 2-2-89 Date

Reviewed by: Otto G. Raabe
Otto G. Raabe, Director, LEHR — 2/28/89 Date

APPENDIX

GCMS Analysis of Fraction 4
Performed at the Lawrence Livermore National Laboratory
Livermore, CA 94550

Biomedical & Environmental Sciences Divisions
Lawrence Livermore National Laboratory

May 30, 1987

Dr. Marvin Goldman
School Of Veterinary Medicine
Department of Veterinary Pharmacology
and Toxicology
University of California
Davis, California 95616

Dear Dr. Goldman:

In April I received from your office two vials containing chemicals isolated by Dr. Alan Buckpitt's research group. The samples were described as high performance liquid chromatography fractions which contained weak mutagen(s) from a munition manufacturing process. It appeared that these samples needed chemical characterization in order to identify the chemical mutagen(s).

Each sample arrived at my laboratory in sealed glass containers and appeared as a light yellow solid residue. To characterize the samples each was analyzed in two different ways. Both methods required computer guided gas chromatography-mass spectrometer (GC-MS) techniques. Each sample revealed a characteristic "fingerprint" of the chemicals. Many types of unique experiments were performed and all analyses were highly specific and very sensitive for the separation and identification of the chemicals present in each sample.

In addition to the isolation, separation, and identification of the organic compounds in each sample, computer data acquisition and data display allowed for select classes of chemicals in each sample to be highlighted. The use of "mass chromatograms" (mass plots) for this purpose is most important in the determination of the chemical similarity between your samples and the identification of trace components in these complex mixtures. Mass chromatogram plots are particularly important to highlight unique classes of substances. In particular, explosives and explosive residues can be detected with mass chromatogram plots.

APPROACH

Initially the HPLC samples (#1 and #2-3) were dissolved in methanol and analyzed to obtain their unique fingerprint of chemical components. The methanol was then evaporated from each of the two samples. A trimethylsilylating (TMS) reagent was then added to the residue, the solution heated, and a small aliquot of the new TMS-derivatized samples analyzed by computer guided GC-MS.

The results of GC-MS analyses will be highlighted below. The figures referred to in the text will be found in the appendix.

The results of both of these analyses is represented in the corresponding total ionization (TI) plots which are similar to a gas chromatographic trace. The chemical profiles reveal time along the x-axis and relative concentration along the y-axis. The longer a chemical takes to appear, the higher its boiling point (and/or polarity). Each peak and shoulder in the TI plot is a single (and different) chemical. Both samples contained many compounds. A large rise in the baseline indicated many substances which were not completely resolved during the chromatographic separation.

I. RESULTS

A. UNDERIVATIZED HPLC FRACTIONS

1. SAMPLE #1 - FRACTION #4

The most prominent component of the TI plot was dimethylsulfoxide. This compound and other trace solvents were observed at 3 minutes into the run. A continuum of minor components were observed during the entire run. The group of compounds seen over 18-19 minutes initially appeared most interesting. However, interpretation of the mass spectral data revealed that the four major components were plasticizers (butyl-benzylphthalate, dioctylphthalates, and dioctylsebacate).

2. SAMPLE #2-3 - FRACTION #4

The analysis of sample #2-3 was very similar to that of sample #1. The DMSO was present and, in addition, dimethylsulfone and 1,3-dioxane were observed. The plasticizers were again seen over the period of 18-19 minutes into the run. A continuum of minor components were also seen over the entire run.

B. TMS-DERVATIZED SAMPLES

In an attempt to derivatize and generate more volatile (and less polar) compounds a trimethylsilating reagent was employed. The TMS ethers and esters which formed were more amenable to gas chromatography.

1. Blank TMS reagent

The total ionization plot of this sample revealed a major impurity, trimethylsilylphosphate at 13 minutes and some minor components which were observed over the period of 8-9 minutes.

2. SAMPLE #1 - TMS

The total ionization plot of the TMS derivatized sample #1 revealed the reagent impurities at 8-9 minutes and the TMS-derivatized phosphate at 13 minutes. Glycine methyl ester (as the N,N-diTMS derivative) is seen at 12 minutes. The plasticizers are seen at 24 minutes. A continuum of compounds are observed over 14-24 minutes.

3. SAMPLE #2-3 - TMS

The total ionization plot of the TMS derivatives from sample #2-3 were more interesting. The TMS reagents impurities were observed at 8-9 and 13 minutes. Glycine (acid, N,O-diTMS derivatize) is seen at 11 minutes, glycine methyl ester at 12 minutes, alpha-hydroxyisocaproic acid-diTMS at 13.9 minutes, a decene-ol (TMS) derivative at 16 minutes, a hydroxy C_{10}-fatty acid at 17.9 minutes, a hydroxy C_{12}-fatty acid at 17.9 minutes, and a hydroxy C_{14}-fatty acid at 18.9 minutes. Many of the minor components appeared to be hydrocarbons and fatty acids.

C. MASS PLOTS

In order to highlight specific compounds in these complex mixtures, mass chromatogram plots were generated. In particular m/z 46 was selected. This fragment ion is highly indicative of NO_2 groups and is often associated with explosive. Mass plots of the parent explosives were also plotted.

The nonderivatized sample #1 revealed a shoulder and a small peak from compounds which generated a fragment ion at m/z 46. This was duplicated in sample #2-3 at 13-14 minutes. Attempts to gain additional data concerning these components failed because each was at low levels in a very complex mixture. The mass spectral data was significantly contaminated with a number of other compounds. This made interpretation of any useful data impossible.

The TMS derivatized sample #1 revealed a trace of a compound buried in the baseline at 19 minutes into the run. The m/z 46 ion was derived from a very minor components and no other useful data concerning this substance could be obtained. Other peaks maximized to highlight m/z 46. However, these compounds did not indicate explosives. The TMS derivatized sample #2-3 did not reveal any compounds with a m/z 46 fragment ions. It would be of interest to know if the Ames assay revealed a lower level of activity for sample #2-3.

DISCUSSION

No conclusive data could be obtained from these runs concerning the identification of a mutagen. This is due to the fact that there are a significant number of contaminating compounds present in each sample. There appeared to be a significant amount of plasticizers and fatty acids from HPLC column packing materials. For the identification of any trace levels of mutagens much cleaner sample will need to be prepared.

We have encountered similar isolation and purity problems during the characterization of food mutagens. Often we have observed an Ames-positive HPLC fraction to possess significant mutagen activity. The HPLC trace would reveal one or often no peaks. However, mass spectral analyses would reveal a signifi-

cant numbers of compounds, complex mixtures and poorly resolved chromatograms. This finding resulted from contaminations and the lack of HPLC purity. It appeared that the food mutagens were present at sub-ppb levels with activities of 500,000 to 600,000 revertants/microgram. Although the fraction initially appeared pure because the revertant/microgram was so high (100's to 1000's of rev/ug-HPLC fraction), the high activity of the compound masked the true purity of the sample.

Therefore, in order to isolate semi-pure material a two column HPLC approach has been followed. We first separated the semi-crude components by reverse phase HPLC. A portion of the collected fractions is tested for AMES activity. Active fractions are then re-chromatographed with a silica column. The new isolated fractions are again tested for mutagen activity. Different HPLC solvent systems are used in each of the chromatographic separations.

This isolation scheme tends to remove contaminants from the mutagenic fractions in the last silica gel column. One class of compounds, the reverse phase HPLC column packing material (fatty acids), can be remove in this process. Also, the HPLC solvent and active HPLC fractions should never come in contact with plastic or materials that have used plastic. Typically an analysis following this type of isolation procedure requires only 100-500 ng (nanograms) of material in order to identify the substance by mass spectrometry.

I hope that these analyses have been helpful. If you would require additional computer print-outs please call me at (415) 422-0903.

Sincerely,

Brian D. Andresen

Brian D. Andresen, Ph.D.

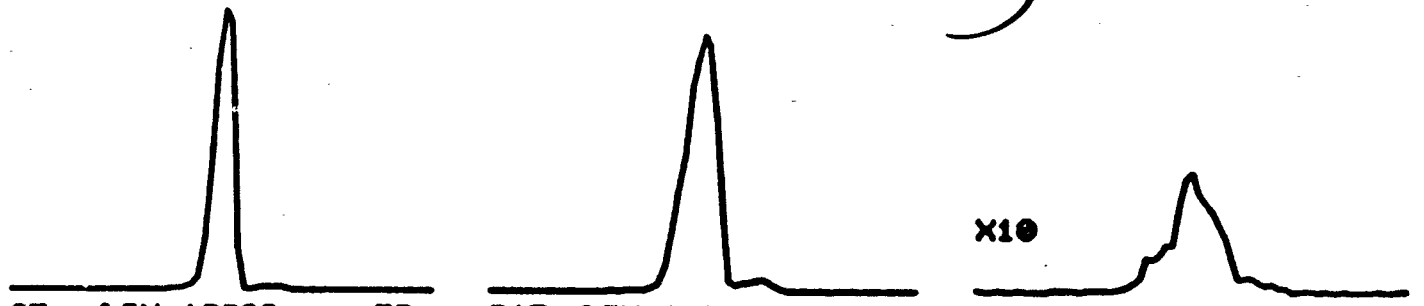
Enc.

Computer Files for Data Acquisition, Recall and Storage for Each Sample

* CURRENT PARAMETERS *
[05985-10041H]

DATE (FRN 1000): 5/9/87 BDA
REPELLER(V)=8.69
DRAWOUT(V)=20
ION FOCUS(V)=6
ENT LENS(MV/AMU)=85
X-RAY(V)=122
EMISSION(UA)=300
ELECT. ENERGY(EV)=70
LOG AMP OFFSET=2
EM VOLTAGE(V)=2400
AMU GAIN=50
AMU OFFSET=95
MASS AXIS GAIN=1.00307
MASS AXIS OFFSET=-1.38483
IONS: POSITIVE
ACTUAL SOURCE TEMP =204

Calibration



ACTUAL MASS	ABS ABUND	REL ABUND	FWHM	ACTUAL ISO MS	ISO ABUND	ACTUAL ISO RATIO	EFF WIDTH
69	12994	100	.44	69.95	201	1.54	.482
219.03	11890	91.5	.69	220.05	515	4.33	.644
502.04	550	4.23	.91	502.89	248	45.09	.742

OPTION?

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PRESENT LIST OF SETPOINT PARAMETERS FOLLOWS:

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TEMP2     400   300
INJ TEMP  400   300   300
FID TEMP  400   300     0
TCD TEMP  400   300   300
AUX TEMP  400   300   278
TIME1       7.00
TIME2      15.00
RATE       10.00
FID SGNL   A
A            0.0    29.3
B            0.0    21.2
PRESS 'CTRL' AND 'R' KEYS TO CONTINUE
>PAUSE
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GC Conditions

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MS TUNING FILE? [ 1000.0]
LAST AUTOTUNE DATE: 5/8/87 BDA

NEW FRN? 5585
CRN? 18
WORDS AVAILABLE ON CRN  18 = 1246848.
APPROXIMATE SCAN TIME (MINUTES) = 692

GC, LC or DIP? GC
EI OR CI? [E]

(1) RUN TIME? 30
(2,3,4) START, STOP MASSES? 45,800
(6) A/D MEASUREMENTS PER DATUM POINT? [    3.0]
(7) THRESHOLD? 15
(8) SCAN START DELAY? 2
(14) ION SOURCE TEMPERATURE? [  200.0]
ELECTRON MULTIPLIER VOLTAGE? [ 2400.0] 2600
SAMPLE NAME?
BUCKPITT (SAMPLE #1 - FRACTION #4) METHANOL SOLUBLE
MISC. DATA?
70/2/10/300 (30M DB1) BDA
SAVE GC REPORT? [N]
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MS TUNING FILE? [ 1000.0]
LAST AUTOTUNE DATE: 5/8/87 BDA

NEW FRN? [ 5585.0] 5586
CRN? [   18.0]
WORDS AVAILABLE ON CRN  18 =  810516.
APPROXIMATE SCAN TIME (MINUTES) = 450

GC, LC or DIP? [G]
EI OR CI? [E]

(1) RUN TIME? [   30.0]
(2,3,4) START, STOP MASSES? [   45.0_  600.0]
(6) A/D MEASUREMENTS PER DATUM POINT? [    3.0]
(7) THRESHOLD? [   15.0]
(8) SCAN START DELAY? [    2.0]
(14) ION SOURCE TEMPERATURE? [  202.0]
ELECTRON MULTIPLIER VOLTAGE? [ 2600.0]
SAMPLE NAME?
BUCKPITT - SAMPLE 2,3 (FRACTION #4) METHANOL SOLUBLE
MISC. DATA?
70/2/10/300 (30M DB1) BBA
SAVE GC REPORT? [N]
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MS TUNING FILE? [ 1000.0]
LAST AUTOTUNE DATE: 5/9/87 BDA

NEW FRN? 5587
CRN? 19
WORDS AVAILABLE ON CRN  19 = 1246848.
APPROXIMATE SCAN TIME (MINUTES) = 692

GC, LC or DIP? GC
EI OR CI? [E]

(1) RUN TIME? 45
(2,3,4) START, STOP MASSES? 45,600
(6) A/D MEASUREMENTS PER DATUM POINT? [    3.0]
(7) THRESHOLD? 15
(8) SCAN START DELAY? 7
(14) ION SOURCE TEMPERATURE? [  200.0]
ELECTRON MULTIPLIER VOLTAGE? [ 2400.0] 2200
SAMPLE NAME?
SAMPLE #1 (FRACTION #4) TMS DERIVATIVE
MISC. DATA?
70/7/10/300 (30M DB1) BDA
SAVE GC REPORT? [N]
```

```
                    ** HP MS DATA AQUIRE **

MS TUNING FILE? [ 1000.0]
LAST AUTOTUNE DATE: 5/9/87 BDA

NEW FRN? [ 5587.0] 5588
CRN? [   19.0]
WORDS AVAILABLE ON CRN  19 = 1069105.
APPROXIMATE SCAN TIME (MINUTES) = 593

GC, LC or DIP? [G]
EI OR CI? [E]

(1) RUN TIME? [   45.0]
(2,3,4) START, STOP MASSES? [   45.0_  600.0]
(6) A/D MEASUREMENTS PER DATUM POINT? [    3.0]
(7) THRESHOLD? [   15.0]
(8) SCAN START DELAY? [    7.0]
(14) ION SOURCE TEMPERATURE? [  204.0]
ELECTRON MULTIPLIER VOLTAGE? [ 2200.0]
SAMPLE NAME?
SAMPLE 2,3 (FRACTION #4) TMS DERIVATIZED
MISC. DATA?
70/7/10/300 (30M DB1) BDA
SAVE GC REPORT? [N]
```

```
                    ** HP MS DATA AQUIRE **

MS TUNING FILE? [ 1000.0]
LAST AUTOTUNE DATE: 5/9/87 BDA

NEW FRN? [ 9999.0]
CRN? [   19.0]
WORDS AVAILABLE ON CRN  19 = 1246848.
APPROXIMATE SCAN TIME (MINUTES) = 692

GC, LC or DIP? [G]
EI OR CI? [E]

(1) RUN TIME? [   45.0]
(2,3,4) START, STOP MASSES? [   45.0_  600.0]
(6) A/D MEASUREMENTS PER DATUM POINT? [    3.0]
(7) THRESHOLD? [   15.0]
(8) SCAN START DELAY? [    7.0]
(14) ION SOURCE TEMPERATURE? [  200.0]
ELECTRON MULTIPLIER VOLTAGE? [ 2200.0]
SAMPLE NAME?
BLANK RUN - TMS REAGENTS ONLY 2 UL
MISC. DATA?
70/7/10/300 (30M DB1) BDA
SAVE GC REPORT? [N]
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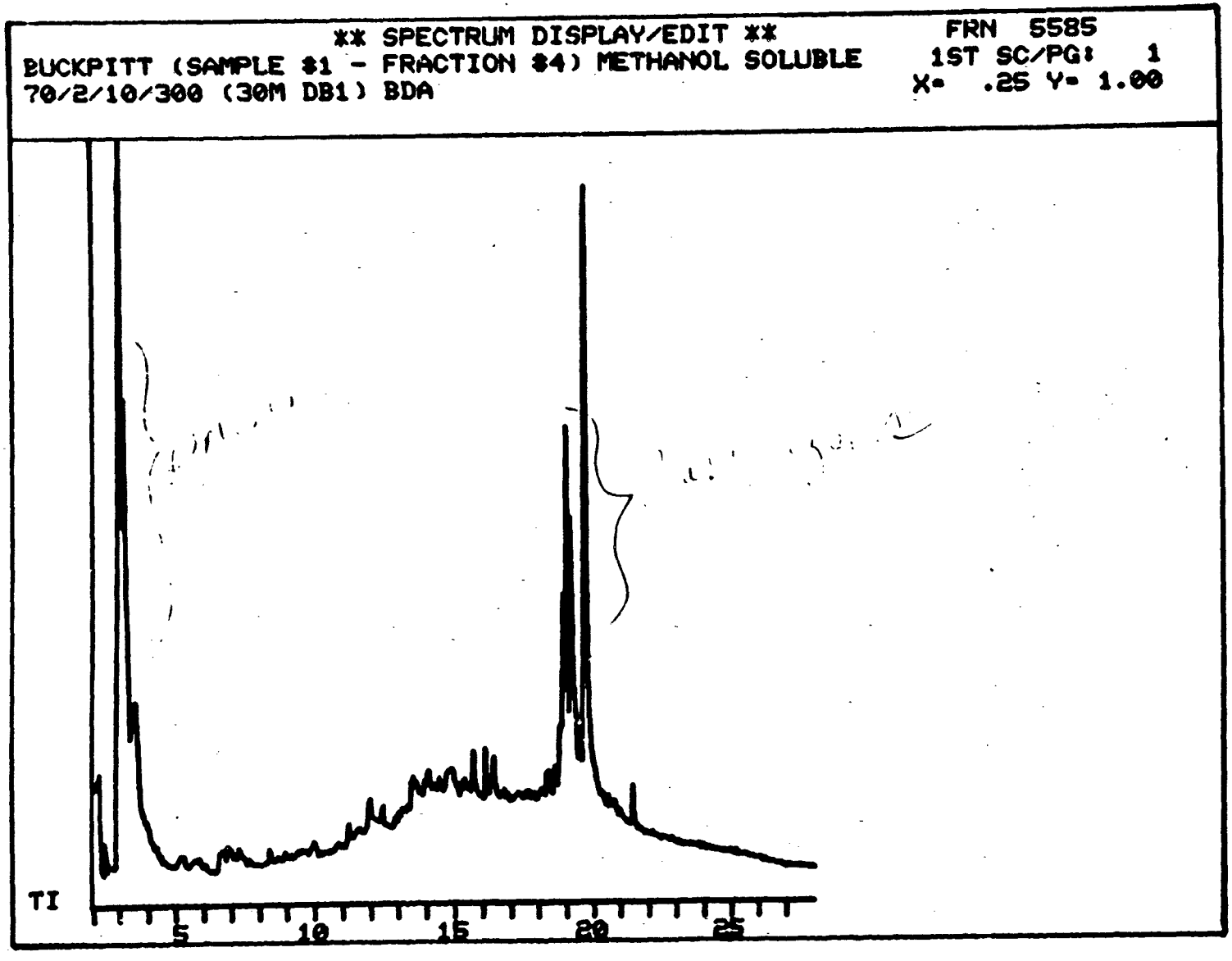
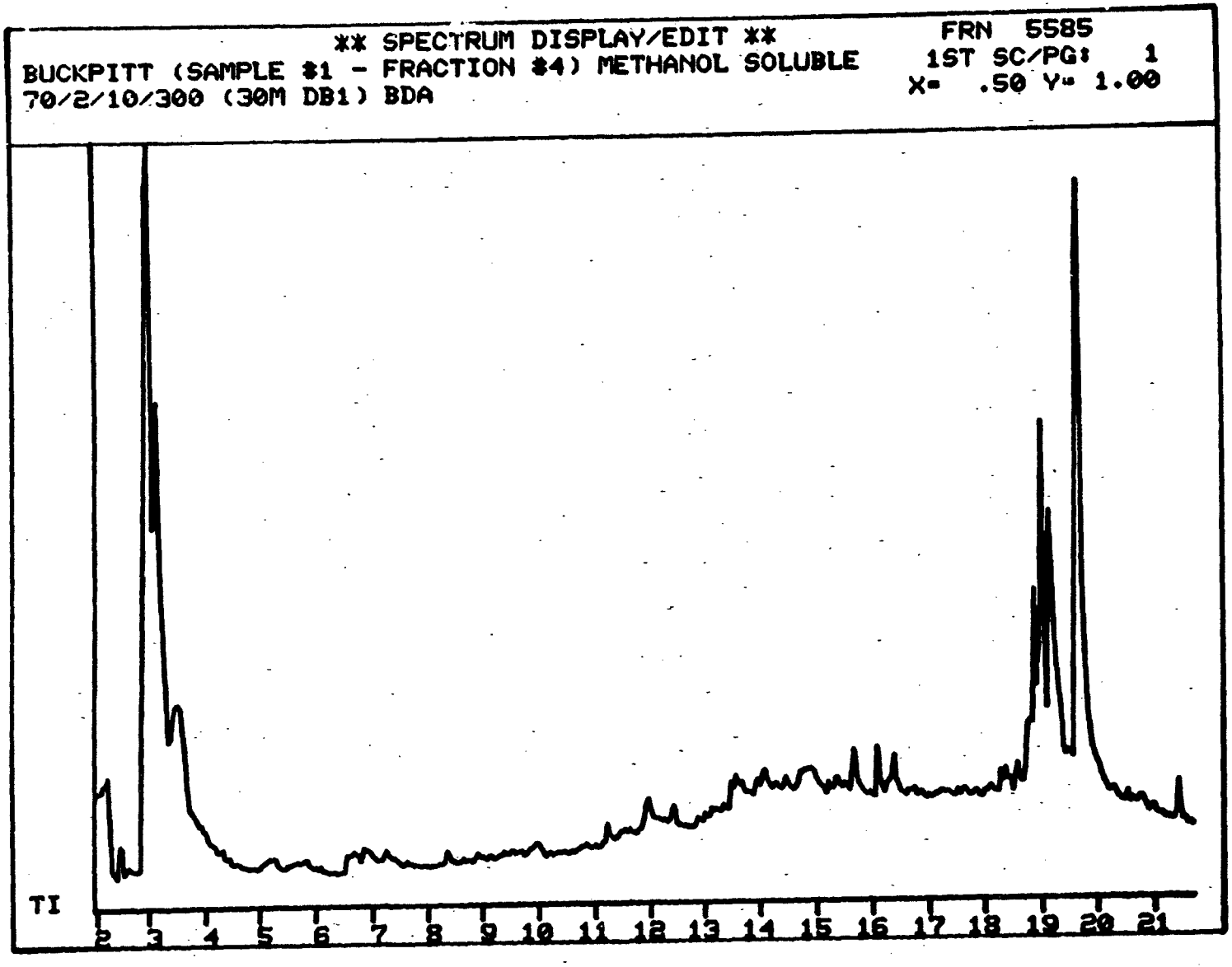
Total Ionization Plots All Compounds In Each Sample

x-Axis = Time y-Axis = Concentration


** SPECTRUM DISPLAY/EDIT **
FRN 5585
BUCKPITT (SAMPLE #1 - FRACTION #4) METHANOL SOLUBLE
1ST SC/PG: 1
70/2/10/300 (30M DB1) BDA
X= .25 Y= 1.00
TI
5
10
15
20
25


** SPECTRUM DISPLAY/EDIT **
FRN 5585
BUCKPITT (SAMPLE #1 - FRACTION #4) METHANOL SOLUBLE
1ST SC/PG: 1
70/2/10/300 (30M DB1) BDA
X= .50 Y= 1.00
TI
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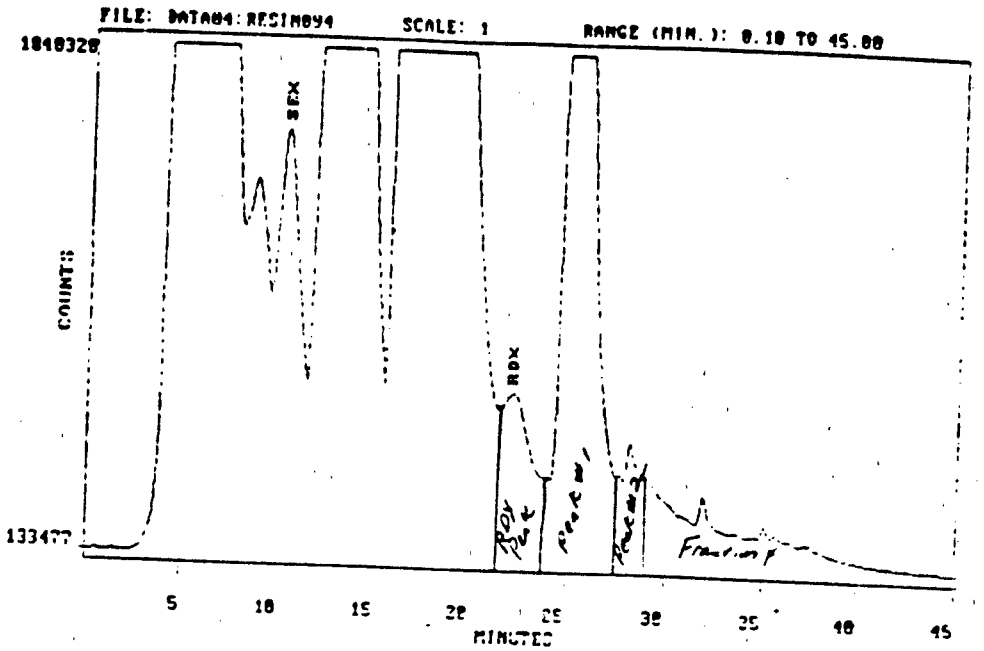
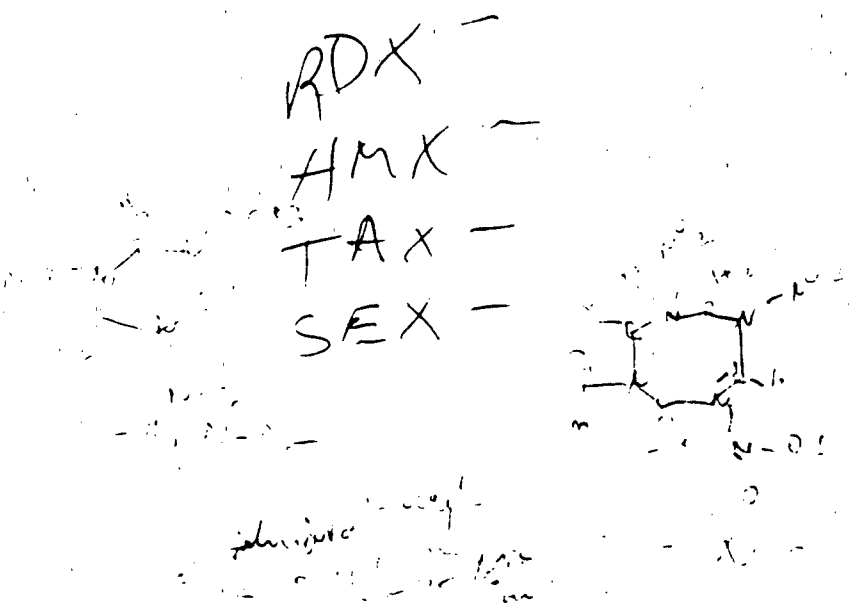
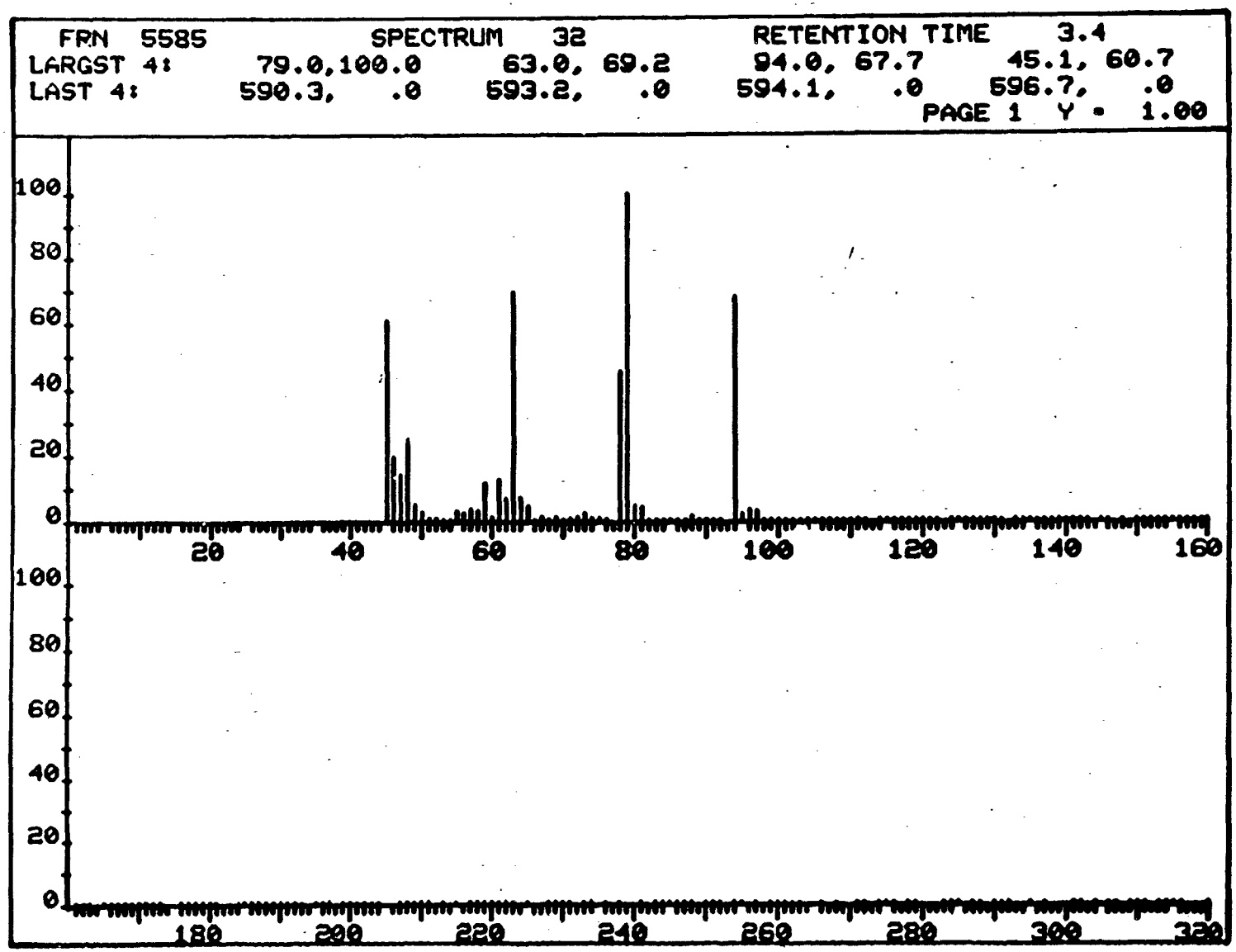


Figure 2. HPLC chromatographic profile (UV at 254 nm) of evaporator sludge indicating the fractions collected for assay using the Ames salmonella tester strain TA 537.



Mass Spectral Data


FRN 5585
SPECTRUM 32
RETENTION TIME 3.4
LARGST 4: 79.0,100.0 63.0, 69.2 94.0, 67.7 45.1, 60.7
LAST 4: 590.3, .0 593.2, .0 594.1, .0 596.7, .0
PAGE 1 Y = 1.00
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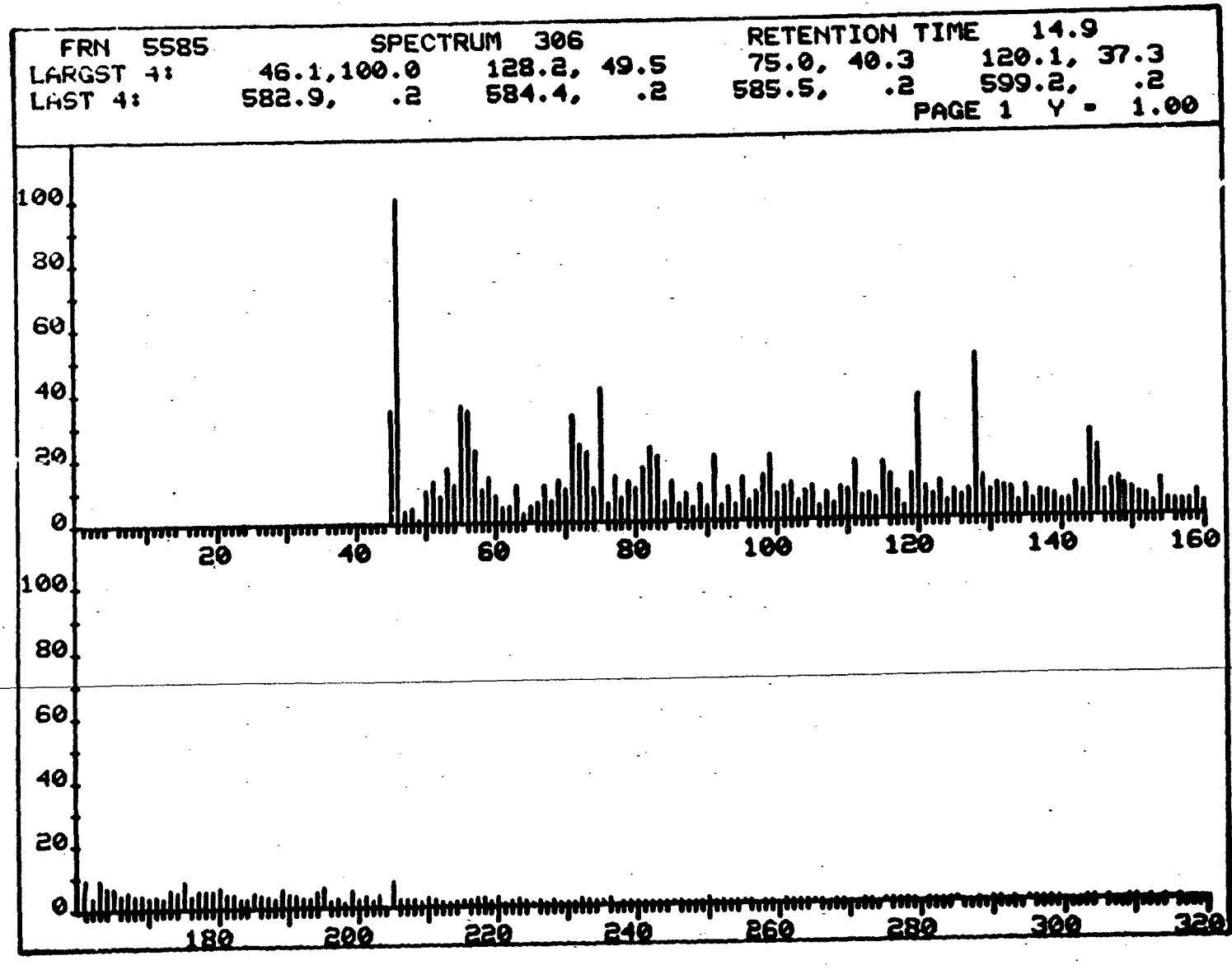
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5585
SPECTRUM 306
RETENTION TIME 14.9
LARGST 4: 46.1,100.0 128.2, 49.5 75.0, 40.3 120.1, 37.3
LAST 4: 582.9, .2 584.4, .2 585.5, .2 599.2, .2
PAGE 1 Y = 1.00
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20 40 60 80 100 120 140 160
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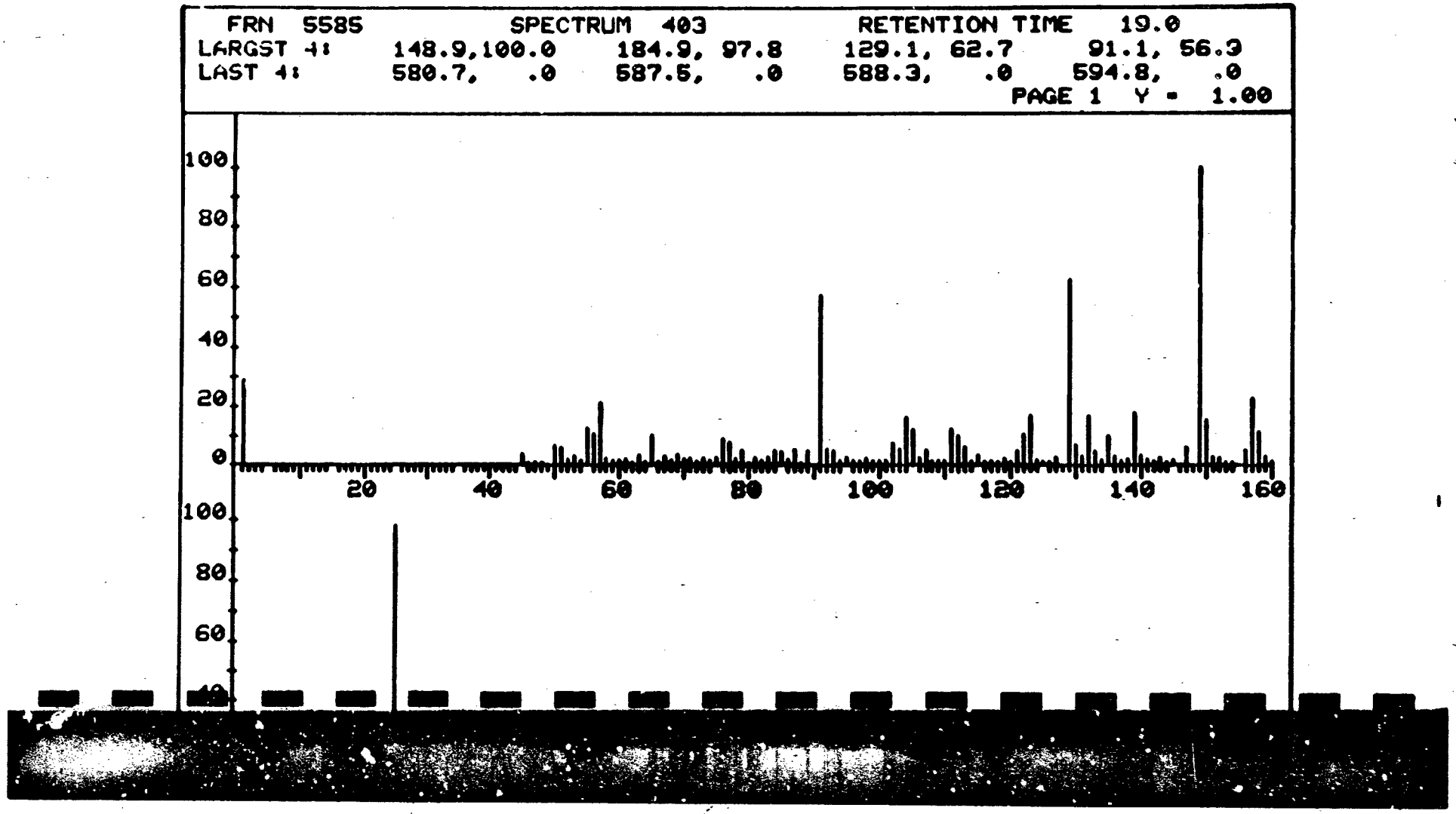
FRN 5585
SPECTRUM 403
RETENTION TIME 19.0
LARGST 4: 148.9,100.0 184.9, 97.8 129.1, 62.7 91.1, 56.3
LAST 4: 580.7, .0 587.5, .0 588.3, .0 594.8, .0
PAGE 1 Y = 1.00
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0
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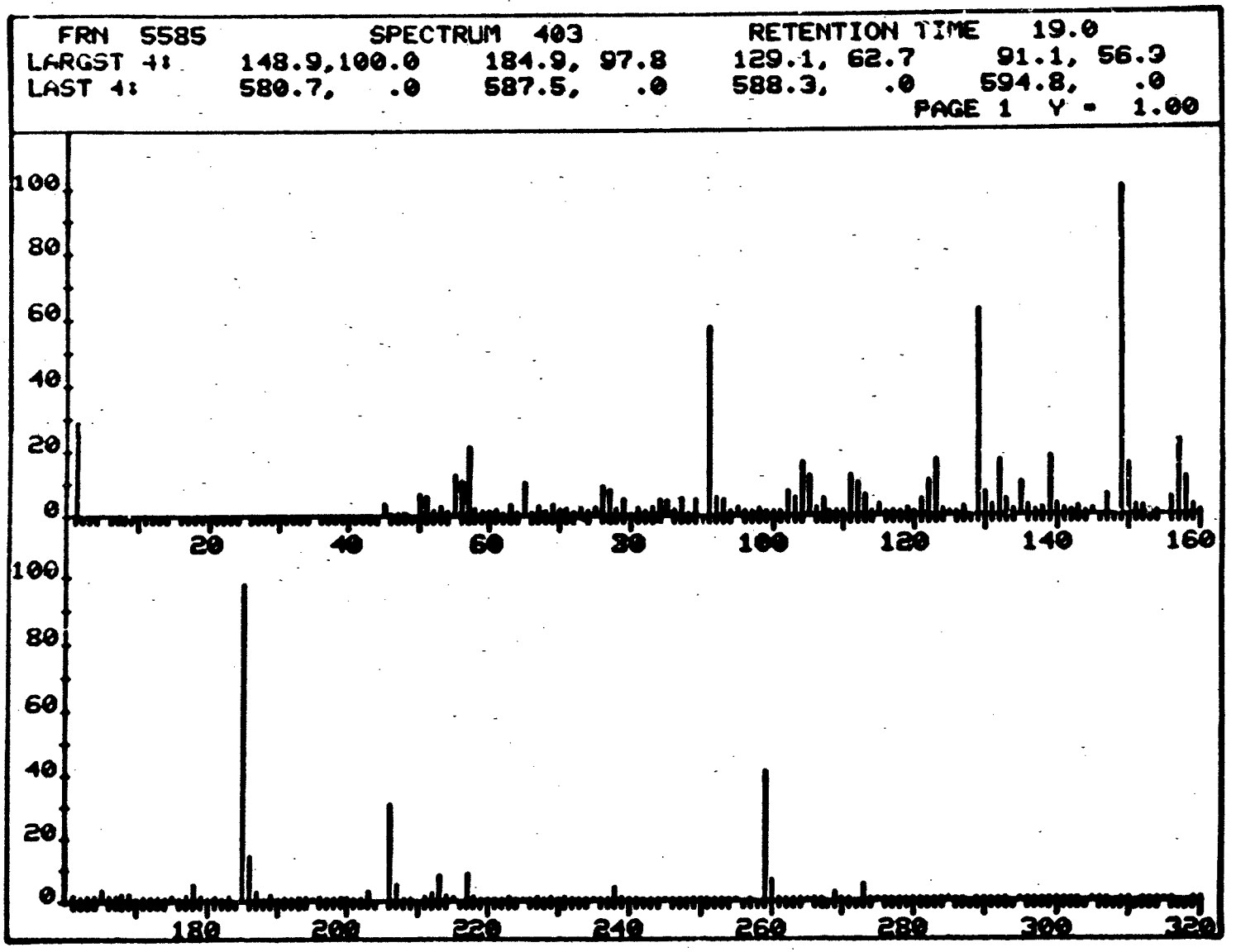
60
80
100
120
140
160
100
80
60


FRN 5585
SPECTRUM 403
RETENTION TIME 19.0
LARGST 4: 148.9,100.0 184.9, 97.8 129.1, 62.7 91.1, 56.9
LAST 4: 580.7, .0 587.5, .0 588.3, .0 594.8, .0
PAGE 1 Y = 1.00
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20 40 60 80 100 120 140 160
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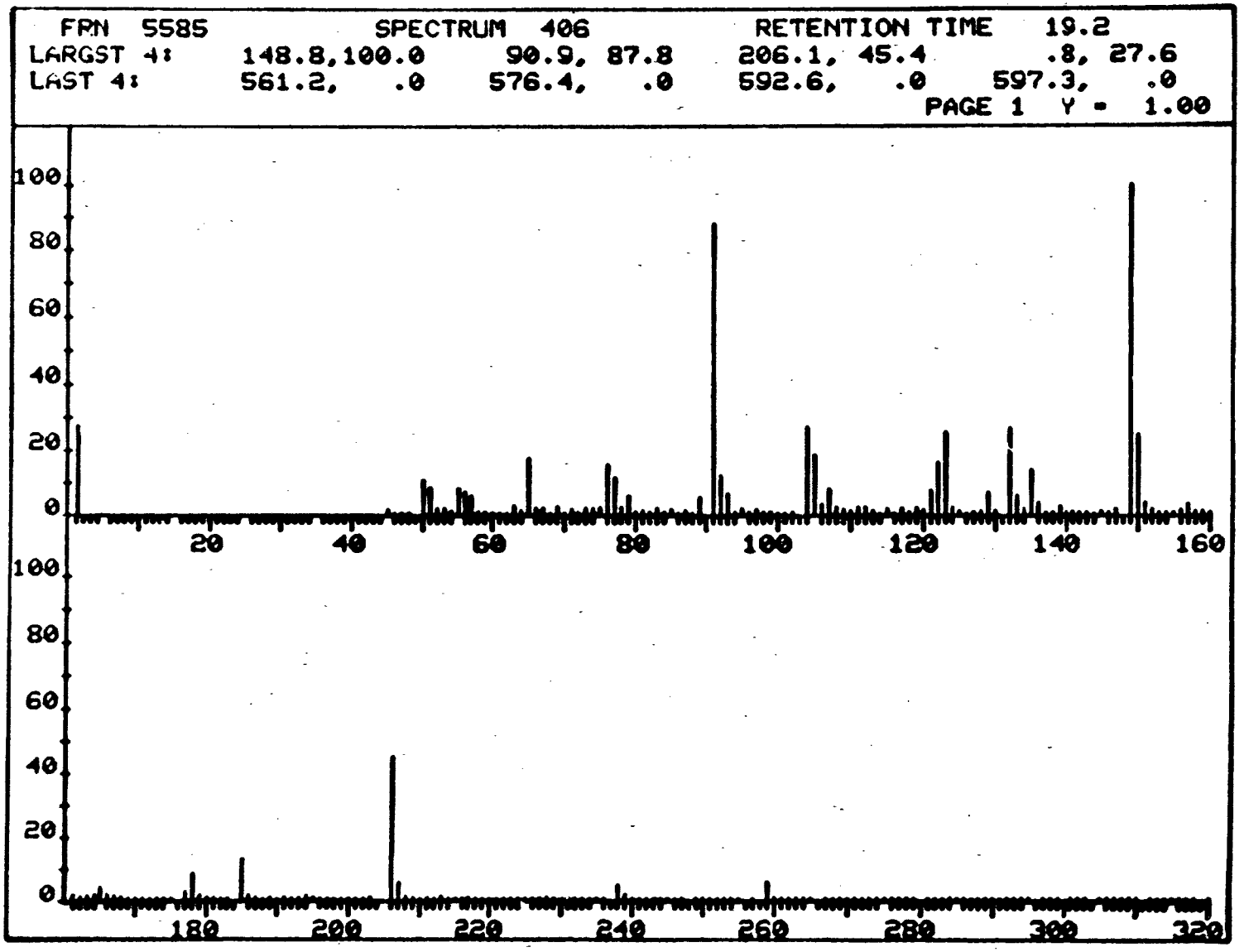
0
180 200 220 240 260 280 300 320


FRN 5585
SPECTRUM 406
RETENTION TIME 19.2
LARGST 4: 148.8,100.0 90.9, 87.8 206.1, 45.4 .8, 27.6
LAST 4: 561.2, .0 576.4, .0 592.6, .0 597.3, .0
PAGE 1 Y = 1.00
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20 40 60 80 100 120 140 160
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80
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40
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0
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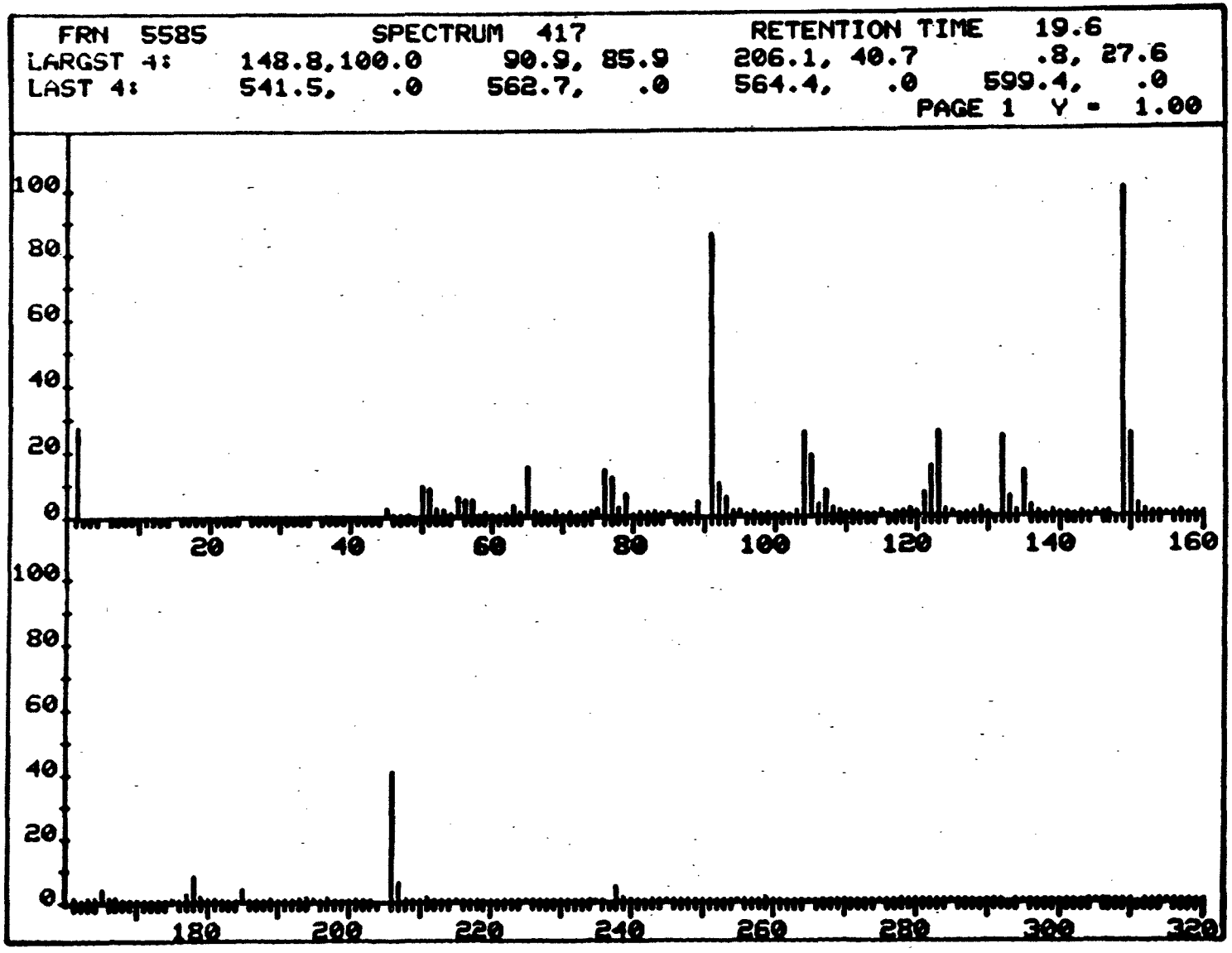
FRN 5585
SPECTRUM 417
RETENTION TIME 19.6
LARGST 4: 148.8,100.0 90.9, 85.9 206.1, 40.7 .8, 27.6
LAST 4: 541.5, .0 562.7, .0 564.4, .0 599.4, .0
PAGE 1 Y = 1.00
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20 40 60 80 100 120 140 160
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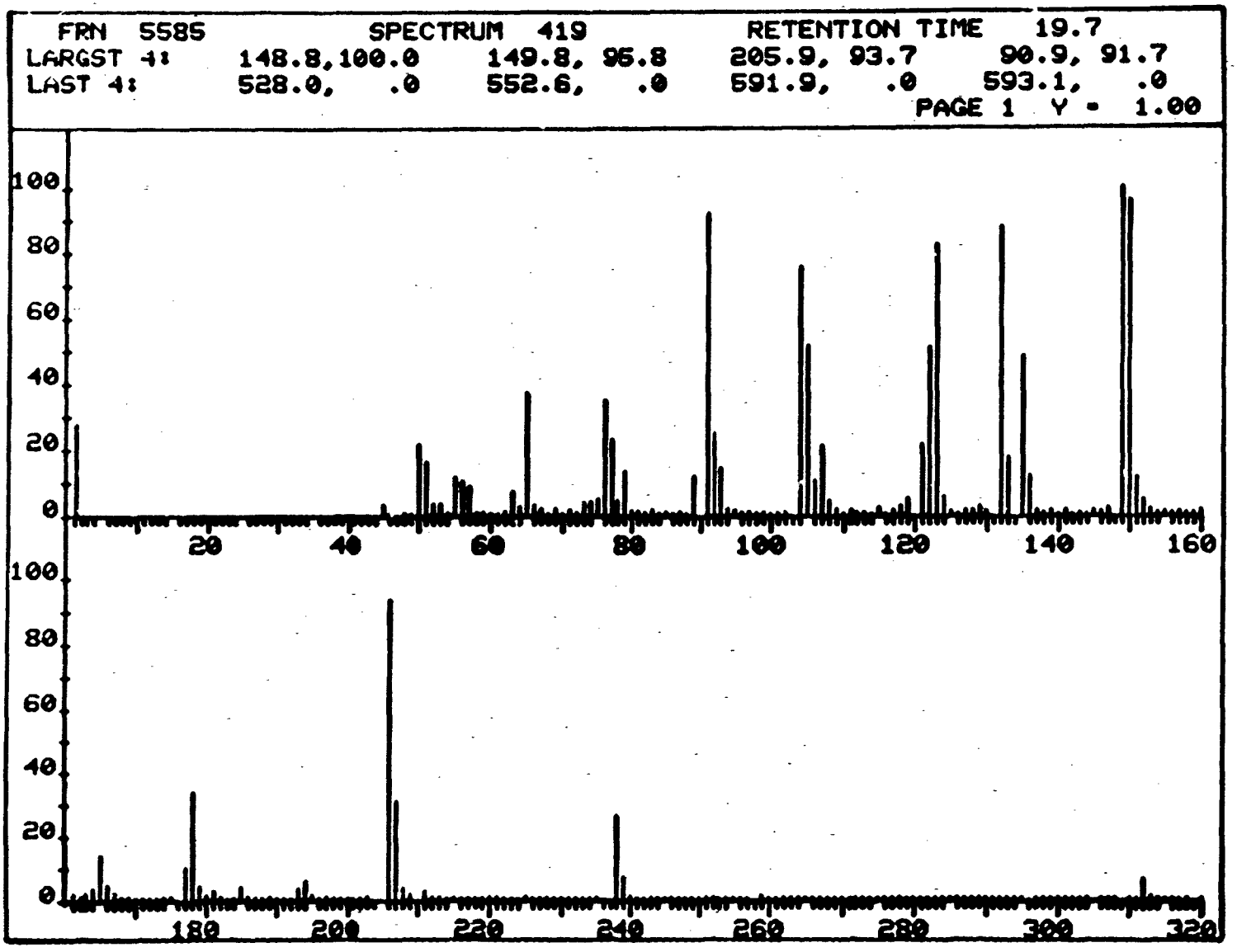
180 200 220 240 260 280 300 320


FRN 5585
SPECTRUM 419
RETENTION TIME 19.7
LARGST 4: 148.8,100.0 149.8, 96.8 205.9, 93.7 90.9, 91.7
LAST 4: 528.0, .0 552.6, .0 591.9, .0 593.1, .0
PAGE 1 Y = 1.00
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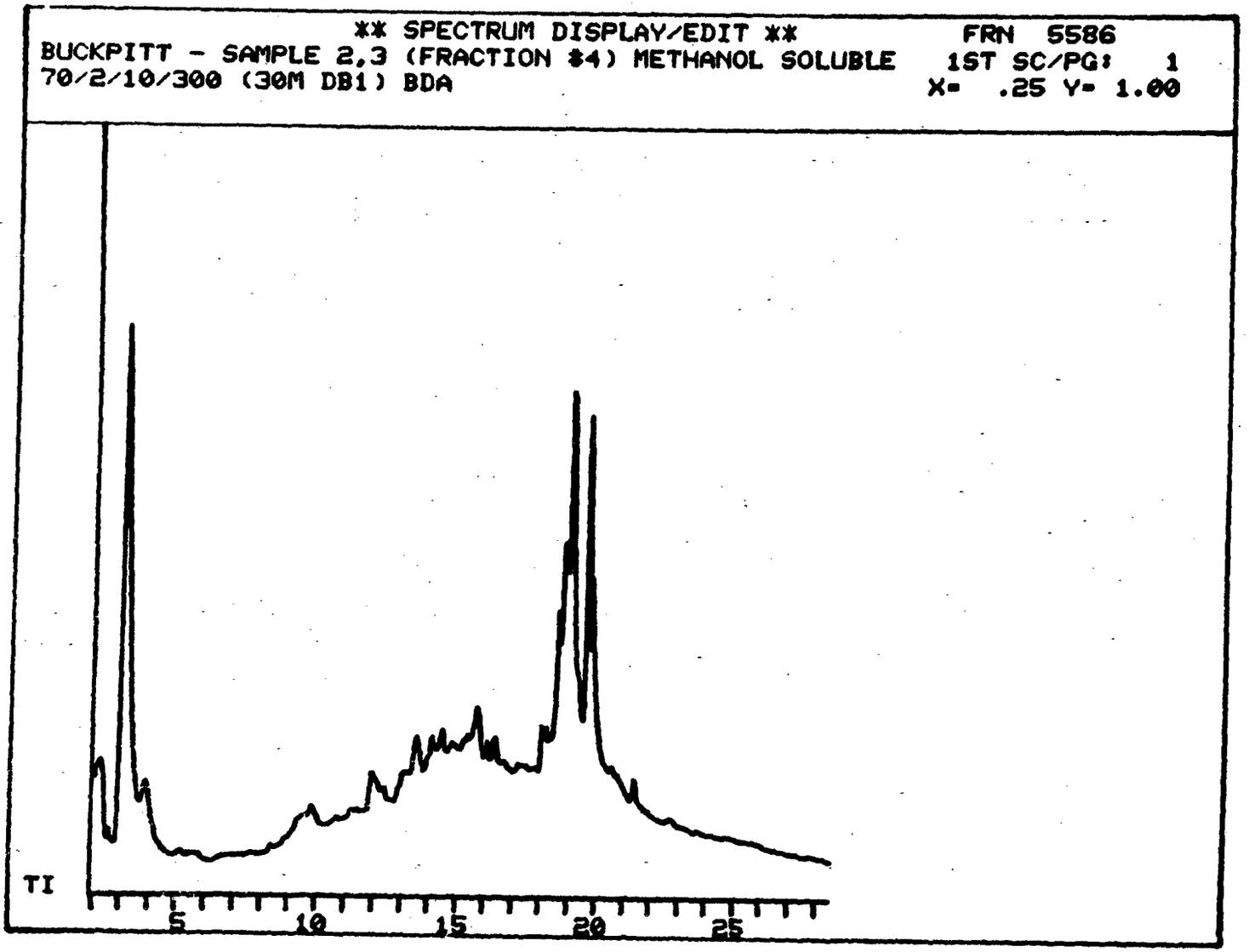
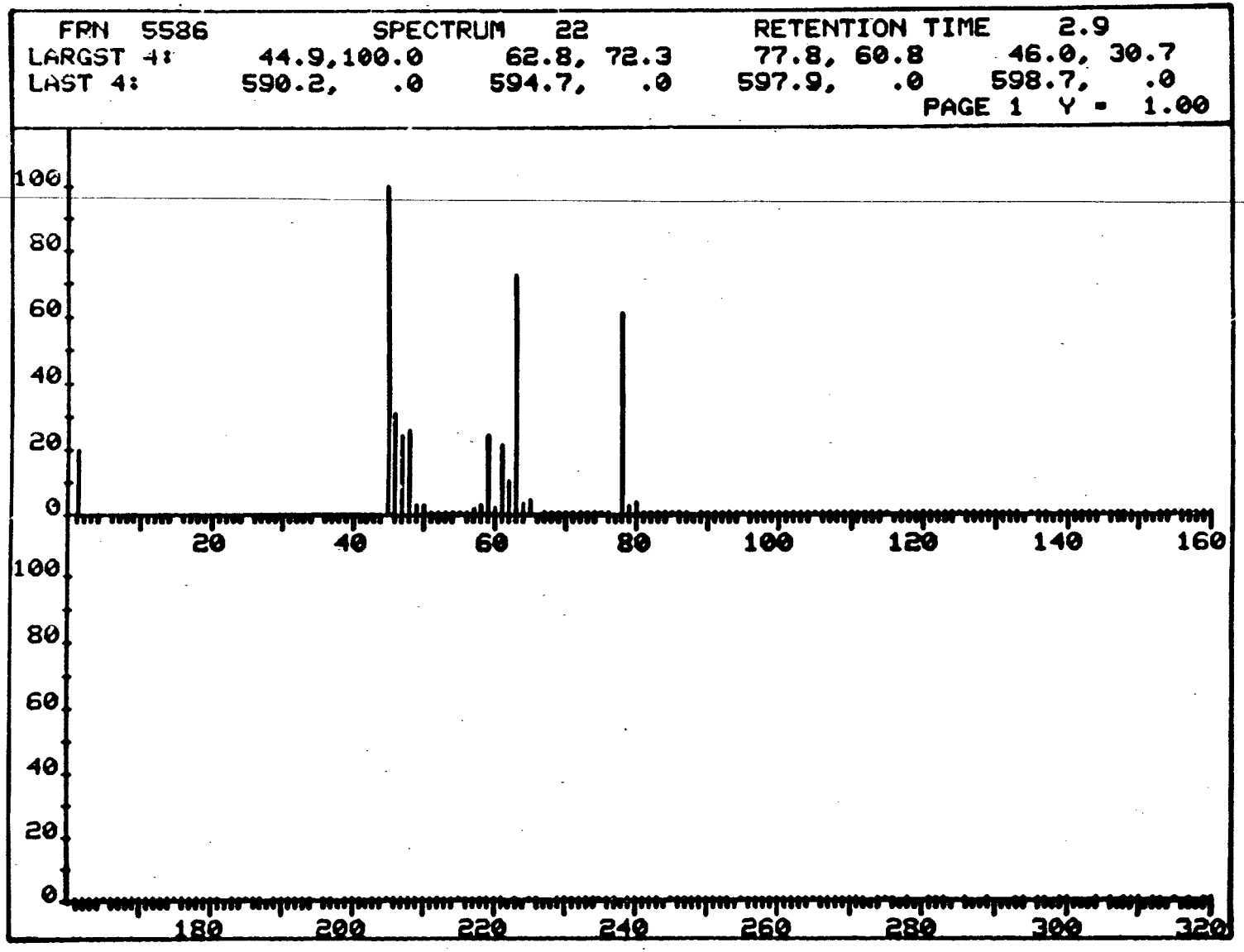

** SPECTRUM DISPLAY/EDIT **
FRN 5586
BUCKPITT - SAMPLE 2,3 (FRACTION #4) METHANOL SOLUBLE
1ST SC/PG: 1
70/2/10/300 (30M DB1) BDA
X= .25 Y= 1.00
TI
5
10
15
20
25


FRN 5586
SPECTRUM 22
RETENTION TIME 2.9
LARGST 4: 44.9,100.0 62.8, 72.3 77.8, 60.8 46.0, 30.7
LAST 4: 590.2, .0 594.7, .0 597.9, .0 598.7, .0
PAGE 1 Y = 1.00
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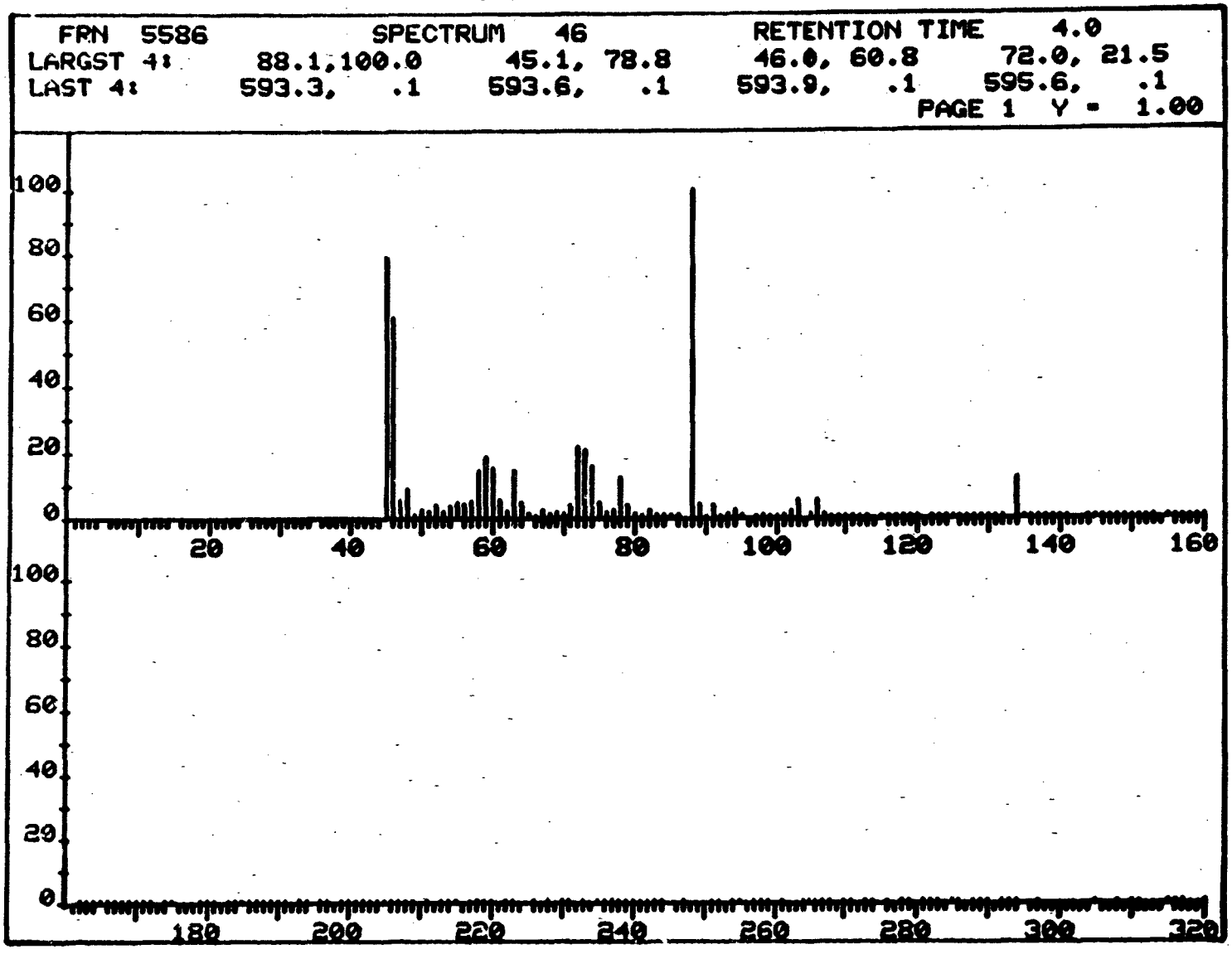
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5586
SPECTRUM 46
RETENTION TIME 4.0
LARGST 4: 88.1, 100.0 45.1, 78.8 46.0, 60.8 72.0, 21.5
LAST 4: 593.3, .1 593.6, .1 593.9, .1 595.6, .1
PAGE 1 Y = 1.00
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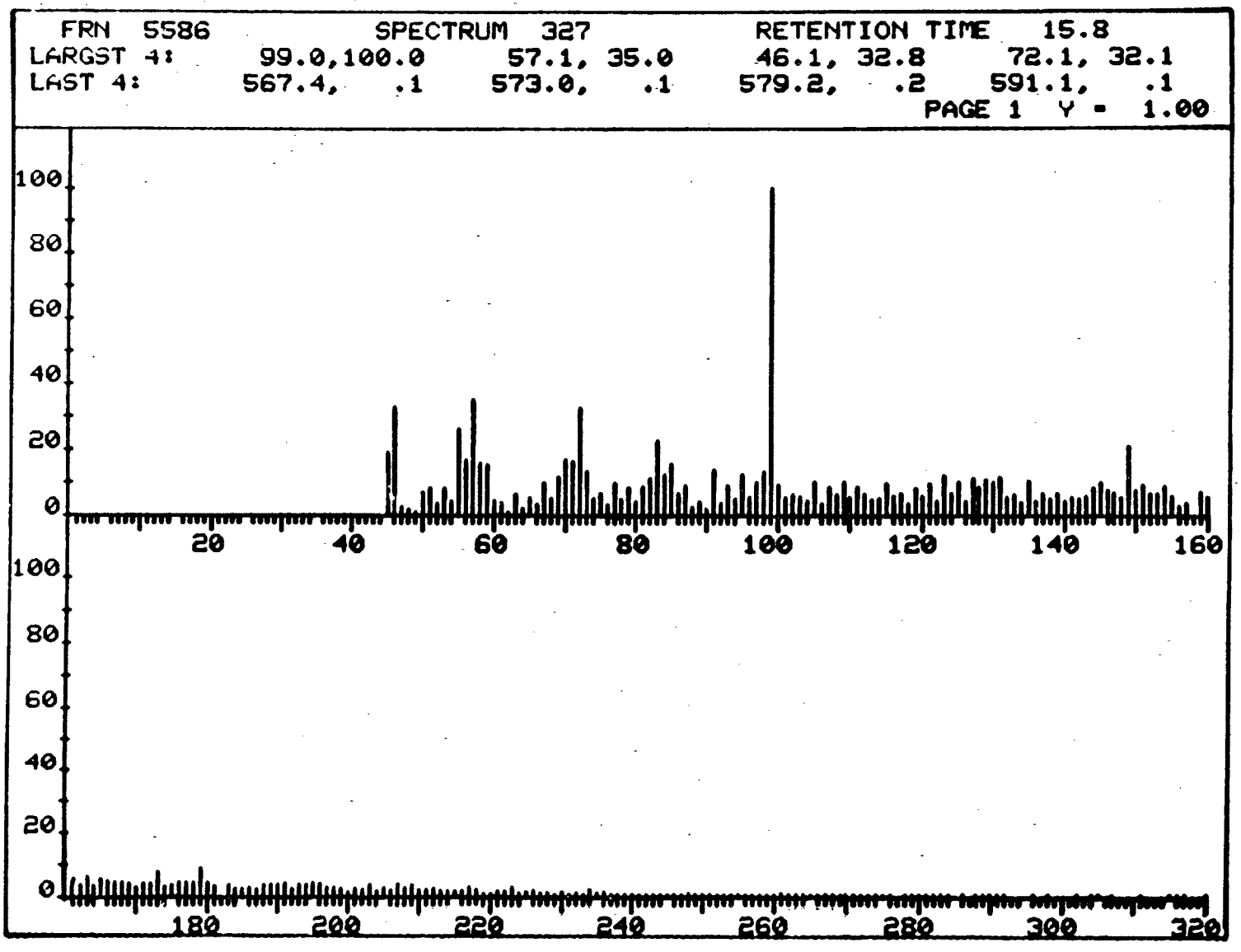
FRN 5586
SPECTRUM 327
RETENTION TIME 15.8
LARGST 4: 99.0,100.0 57.1, 35.0 46.1, 32.8 72.1, 32.1
LAST 4: 567.4, .1 573.0, .1 579.2, .2 591.1, .1
PAGE 1 Y = 1.00
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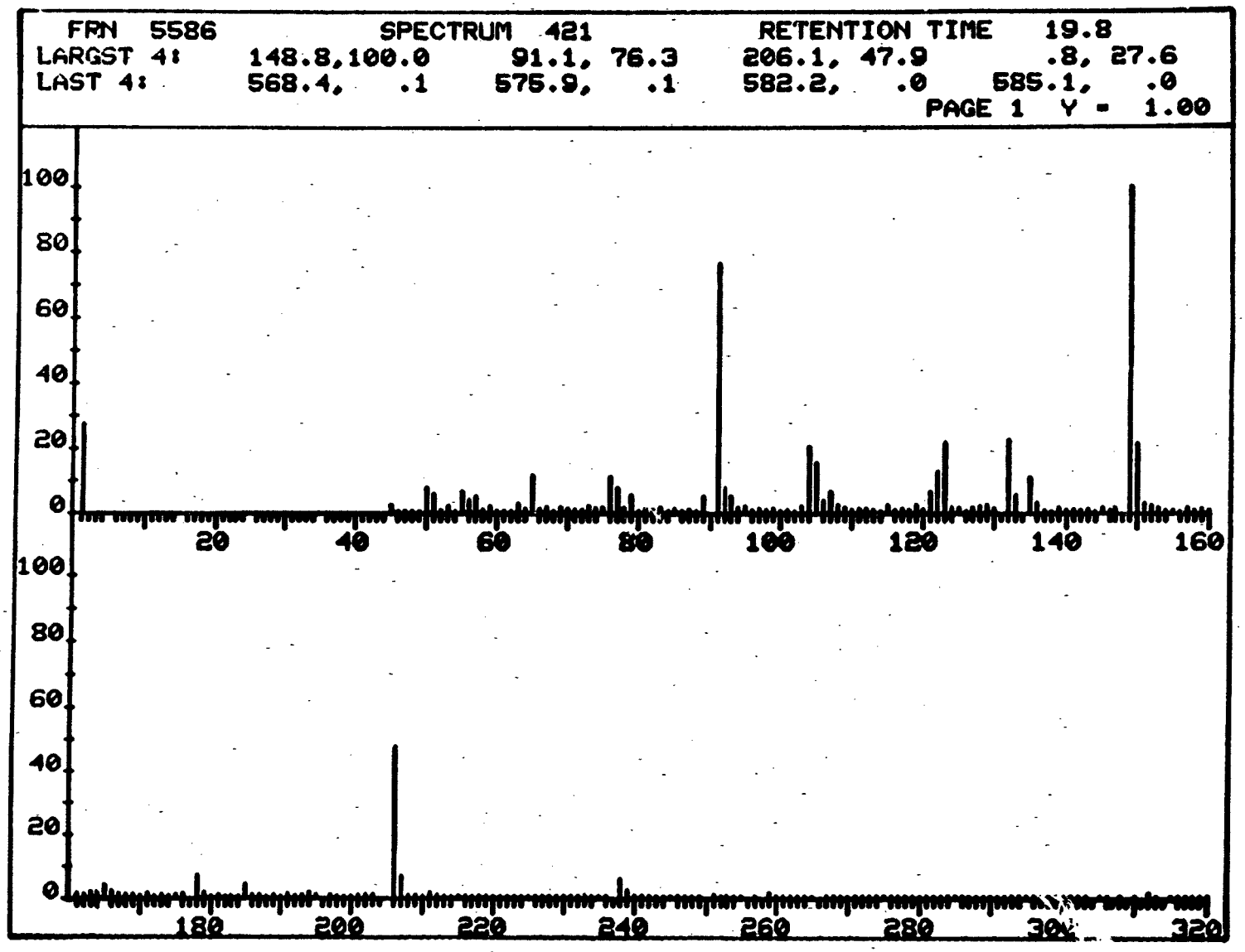
FRN 5586
SPECTRUM 421
RETENTION TIME 19.8
LARGST 4: 148.8,100.0 91.1, 76.3 206.1, 47.9 .8, 27.6
LAST 4: 568.4, .1 575.9, .1 582.2, .0 585.1, .0
PAGE 1 Y = 1.00
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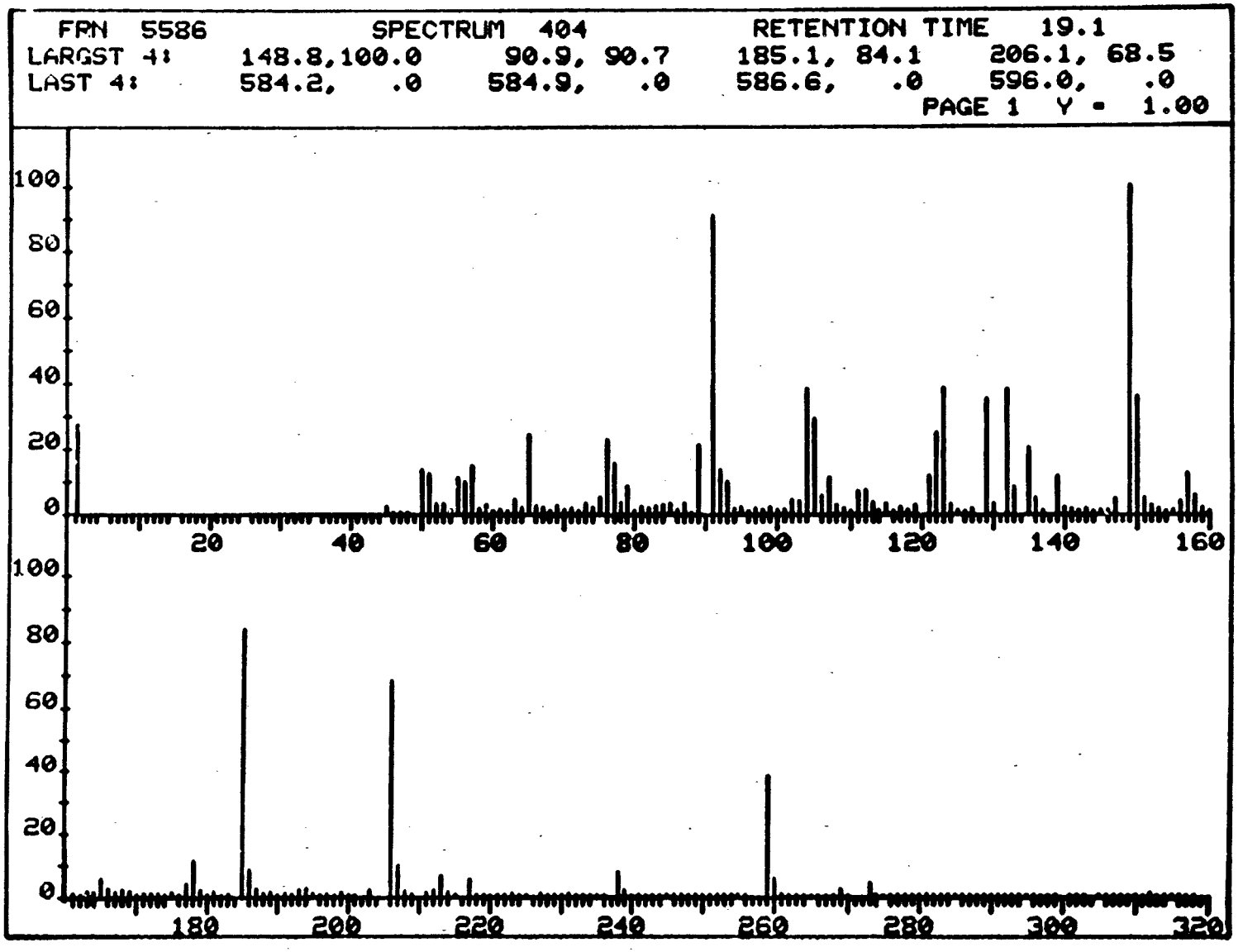
FRN 5586
SPECTRUM 404
RETENTION TIME 19.1
LARGST 4: 148.8,100.0 90.9, 90.7 185.1, 84.1 206.1, 68.5
LAST 4: 584.2, .0 584.9, .0 586.6, .0 596.0, .0
PAGE 1 Y = 1.00
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300
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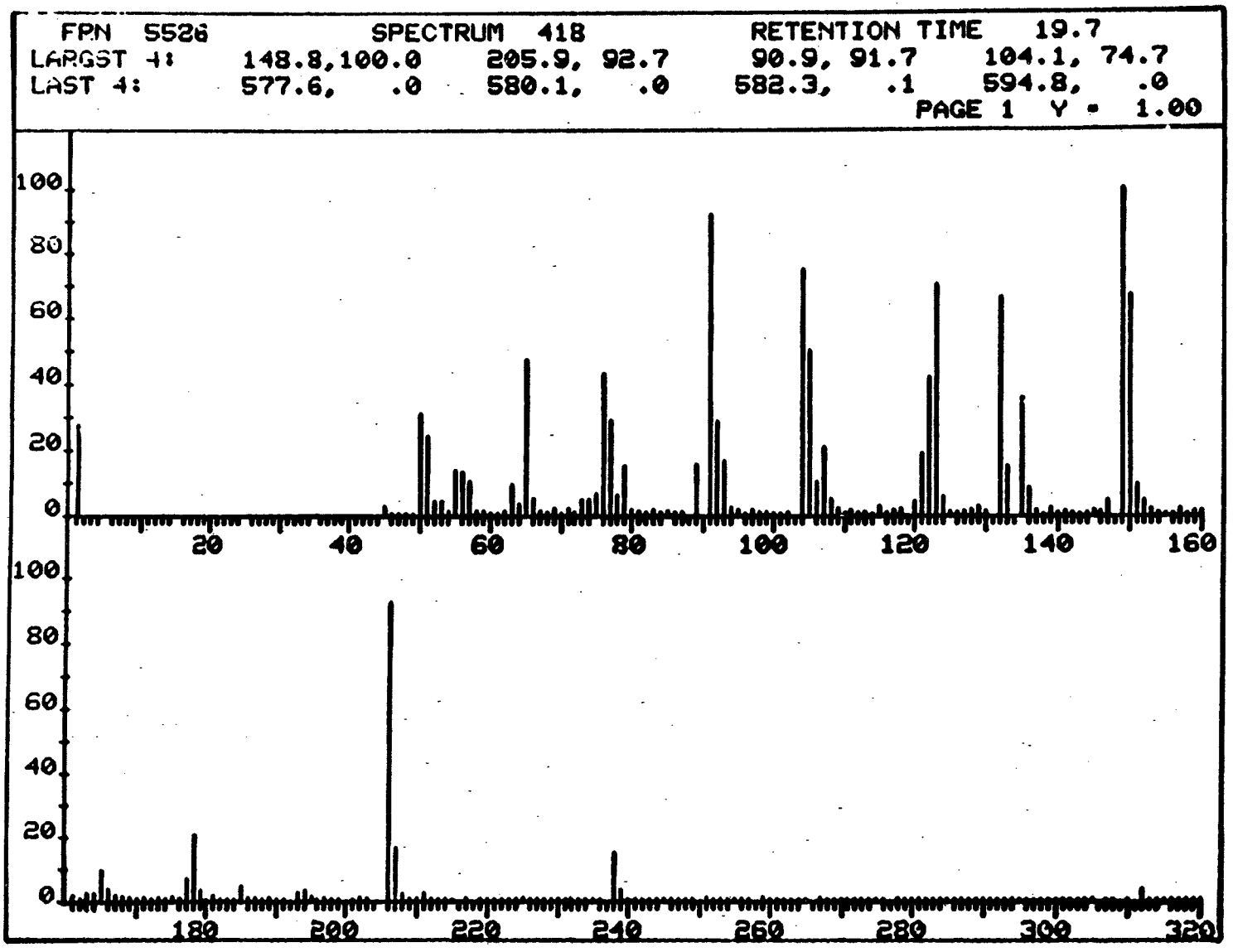
FRN 5526
SPECTRUM 418
RETENTION TIME 19.7
LARGST 4: 148.8,100.0 205.9, 92.7 90.9, 91.7 104.1, 74.7
LAST 4: 577.6, .0 580.1, .0 582.3, .1 594.8, .0
PAGE 1 Y = 1.00
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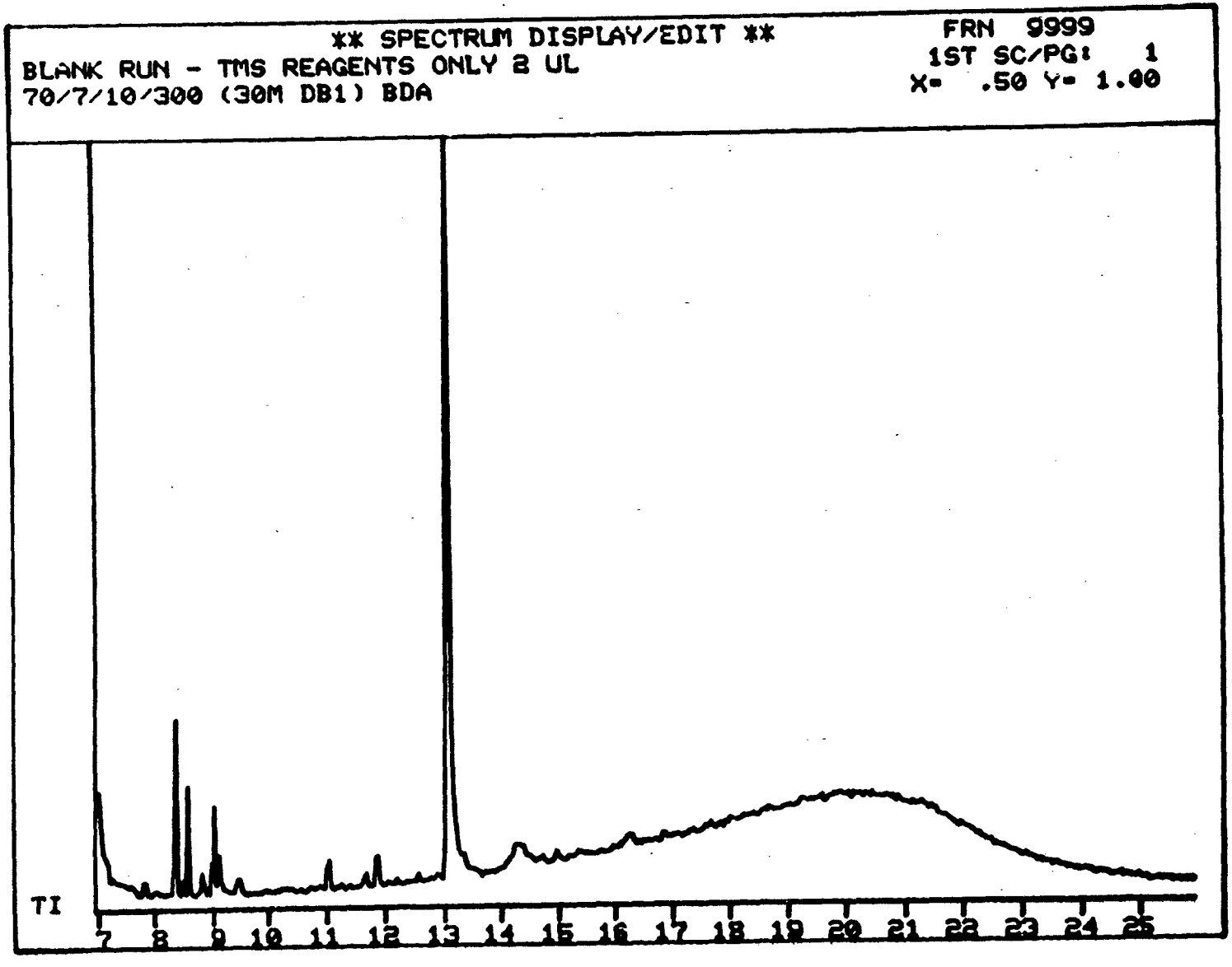
** SPECTRUM DISPLAY/EDIT **
FRN 9999
1ST SC/PG: 1
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BLANK RUN - TMS REAGENTS ONLY 2 UL
70/7/10/300 (30M DB1) BDA
TI
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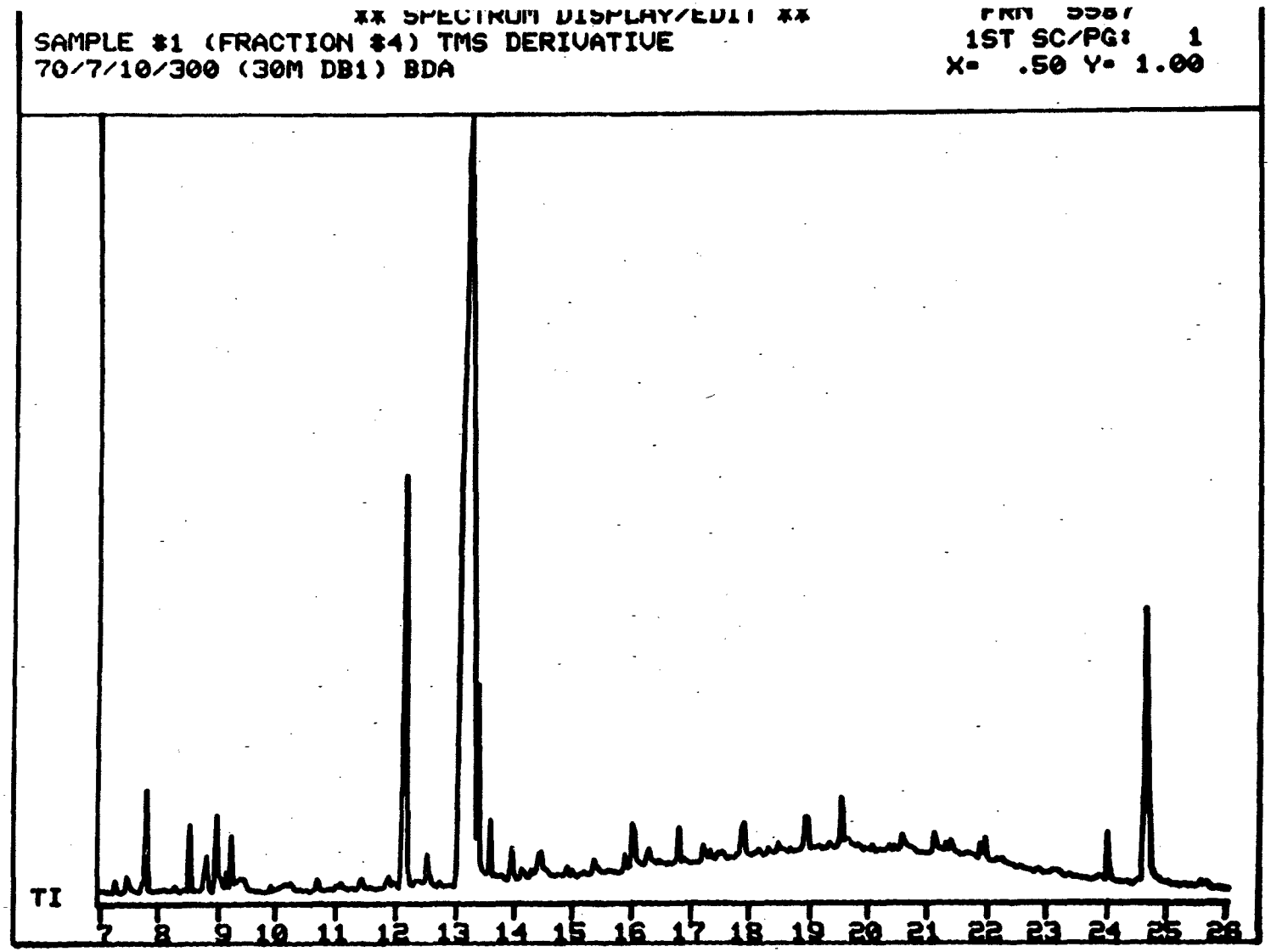
** SPECTRUM DISPLAY/EDIT **
SAMPLE #1 (FRACTION #4) TMS DERIVATIVE
70/7/10/300 (30M DB1) BDA
1ST SC/PG: 1
X= .50 Y= 1.00
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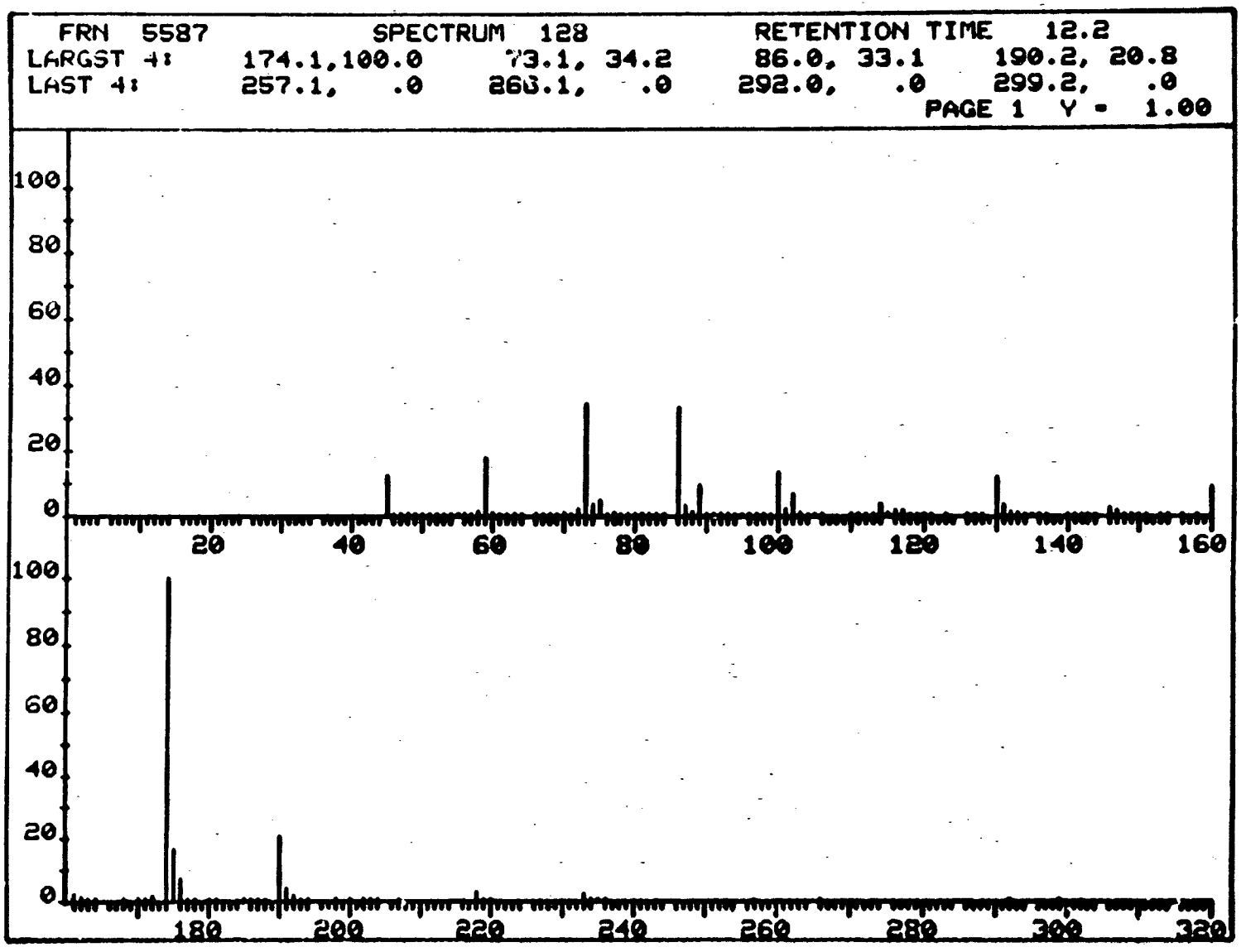
FRN 5587
SPECTRUM 128
RETENTION TIME 12.2
LARGST 4: 174.1,100.0 73.1, 34.2 86.0, 33.1 190.2, 20.8
LAST 4: 257.1, .0 260.1, .0 292.0, .0 299.2, .0
PAGE 1 Y = 1.00
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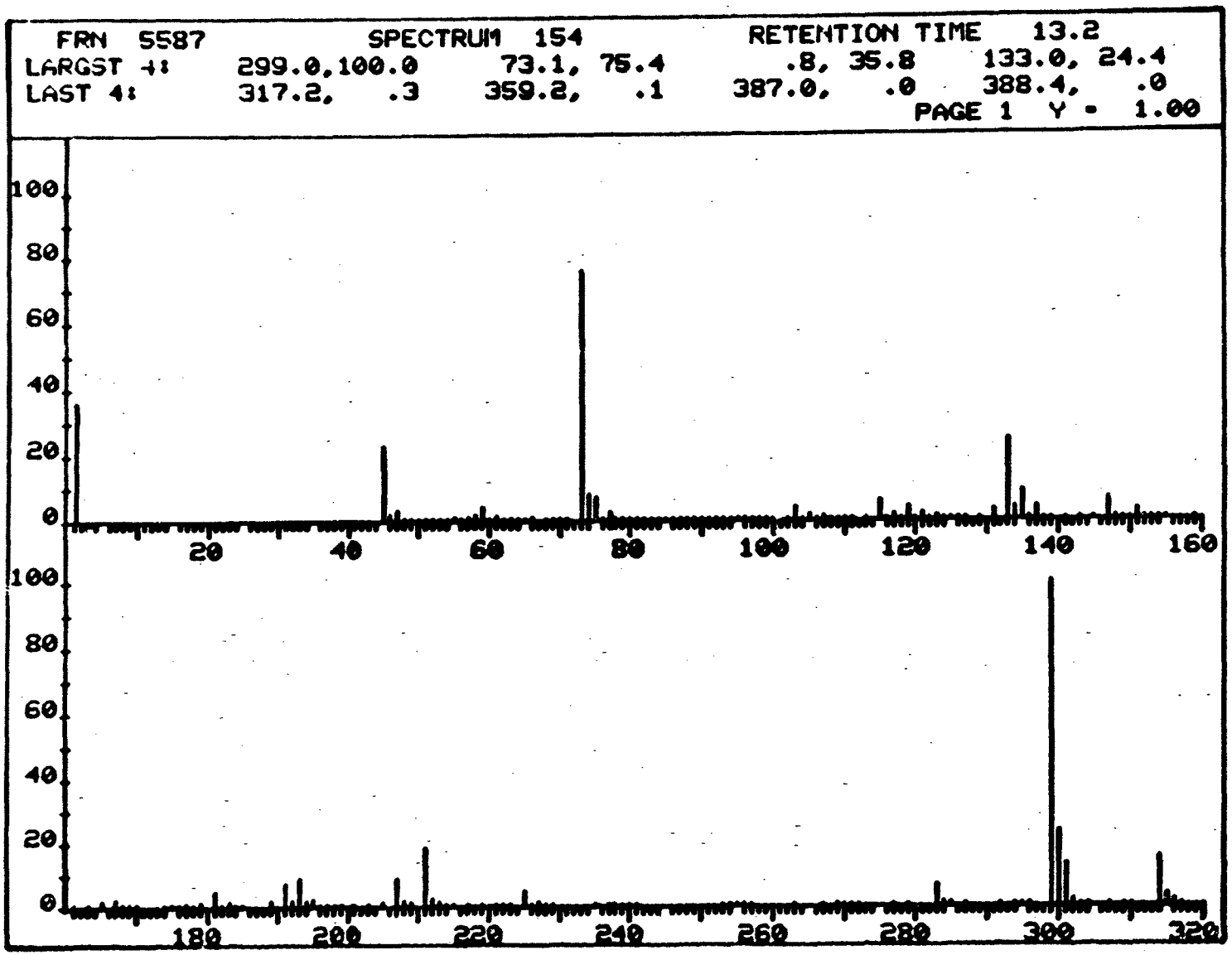
FRN 5587
SPECTRUM 154
RETENTION TIME 13.2
LARGST 4: 299.0,100.0 73.1, 75.4 .8, 35.8 133.0, 24.4
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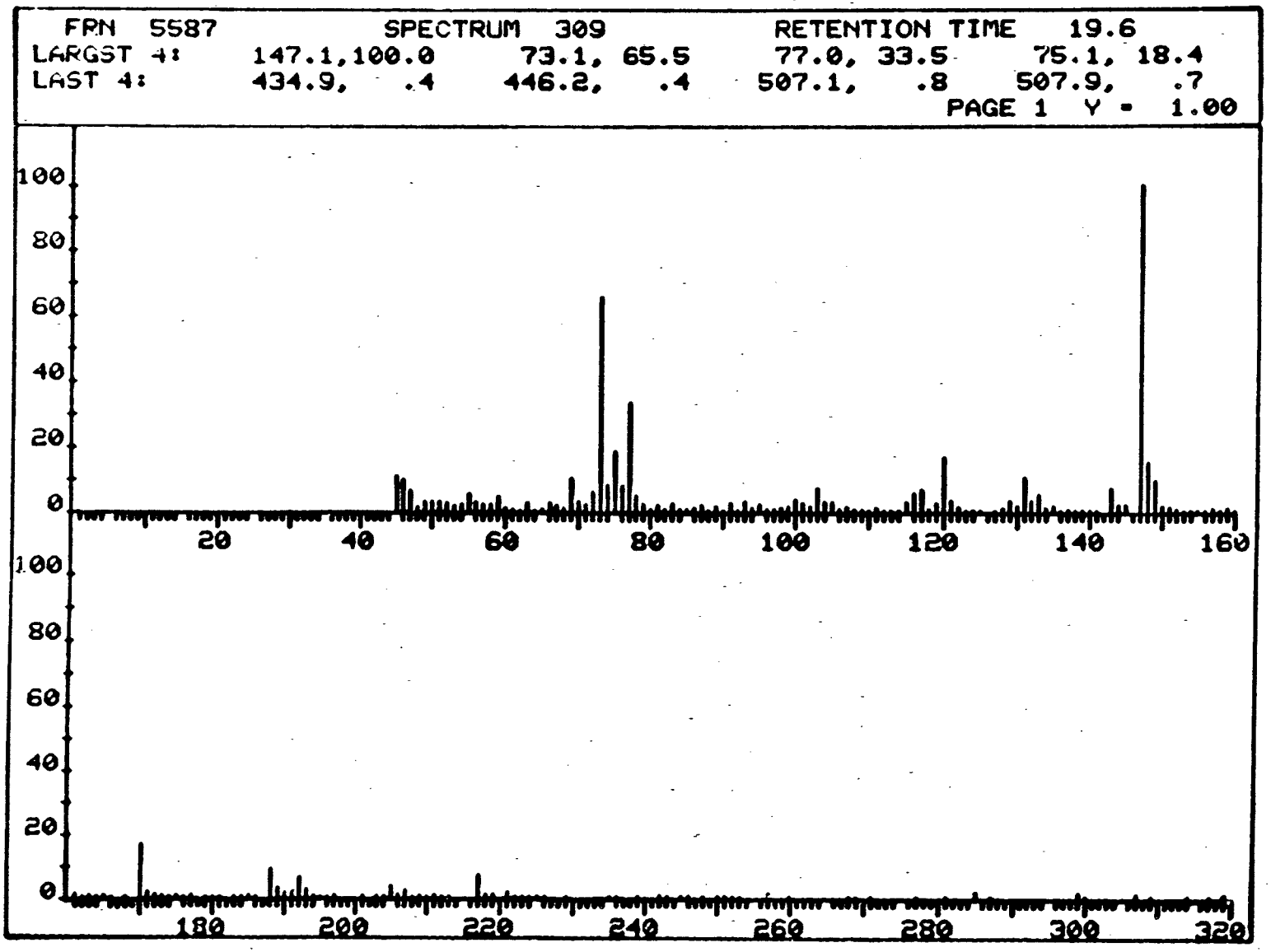
FRN 5587
SPECTRUM 309
RETENTION TIME 19.6
LARGST 4: 147.1,100.0 73.1, 65.5 77.0, 33.5 75.1, 18.4
LAST 4: 434.9, .4 446.2, .4 507.1, .8 507.9, .7
PAGE 1 Y = 1.00
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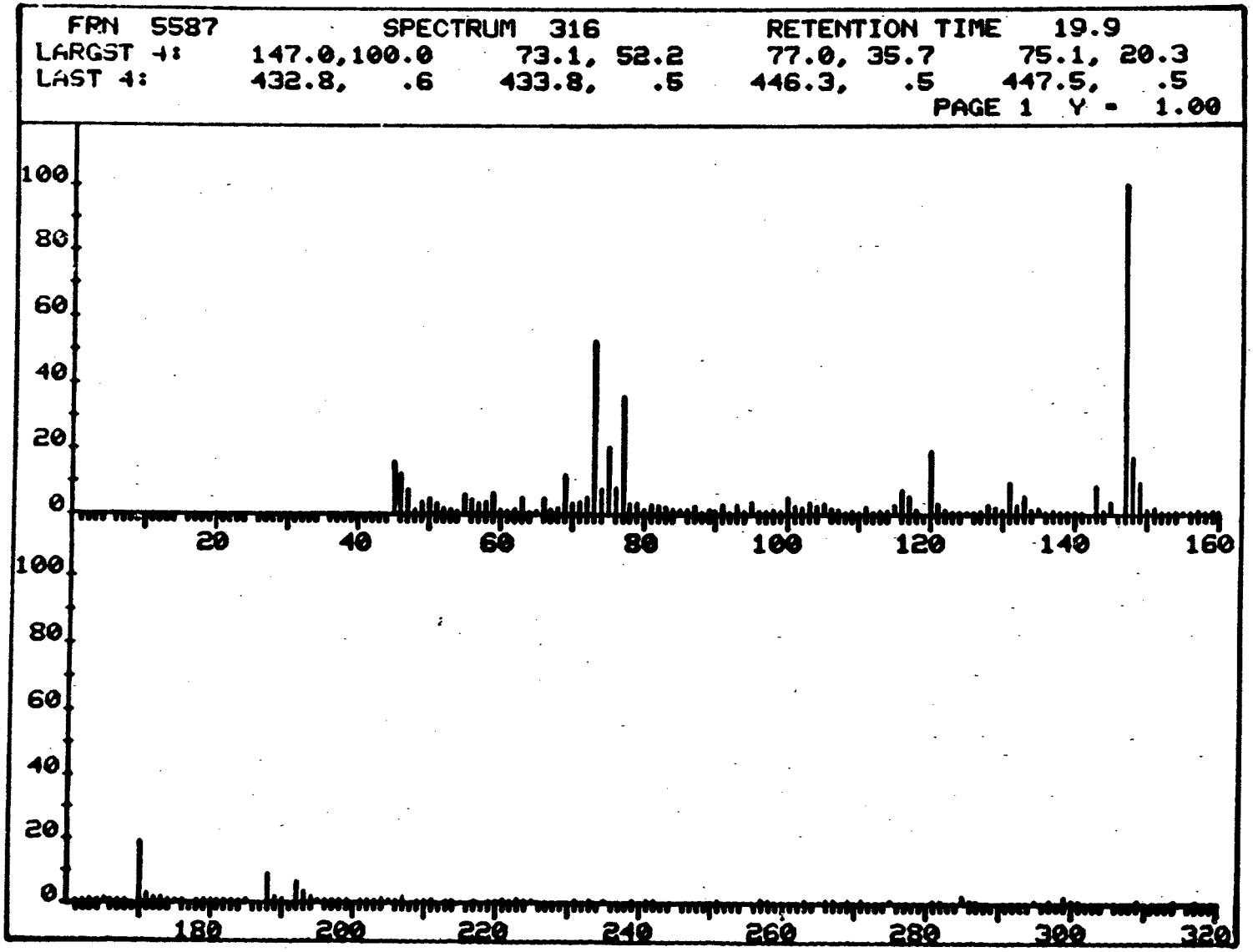
FRN 5587
SPECTRUM 316
RETENTION TIME 19.9
LARGST 4: 147.0,100.0 73.1, 52.2 77.0, 35.7 75.1, 20.3
LAST 4: 432.8, .6 433.8, .5 446.3, .5 447.5, .5
PAGE 1 Y = 1.00
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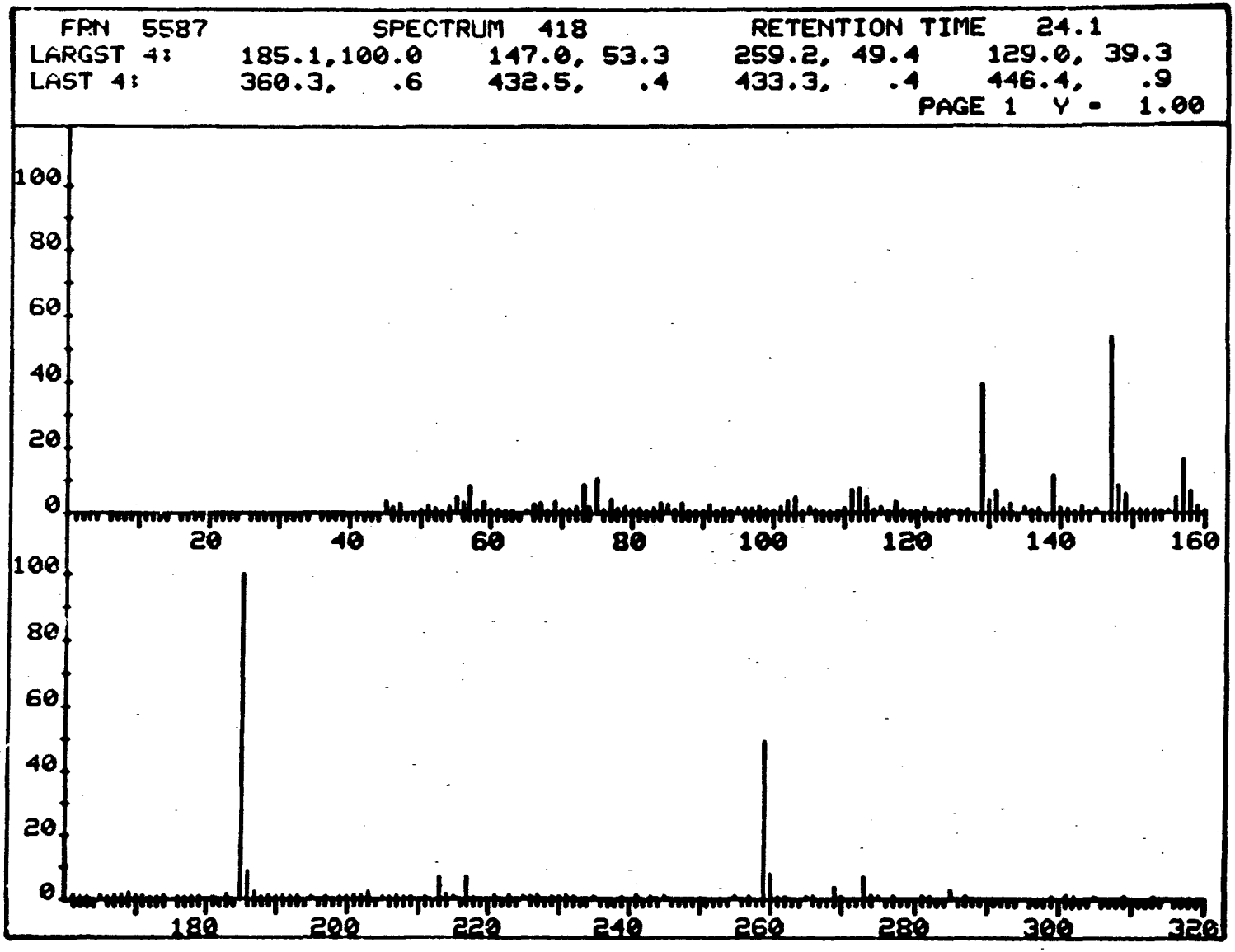
FRN 5587
SPECTRUM 418
RETENTION TIME 24.1
LARGST 4: 185.1, 100.0 147.0, 53.3 259.2, 49.4 129.0, 39.3
LAST 4: 360.3, .6 432.5, .4 433.3, .4 446.4, .9
PAGE 1 Y = 1.00
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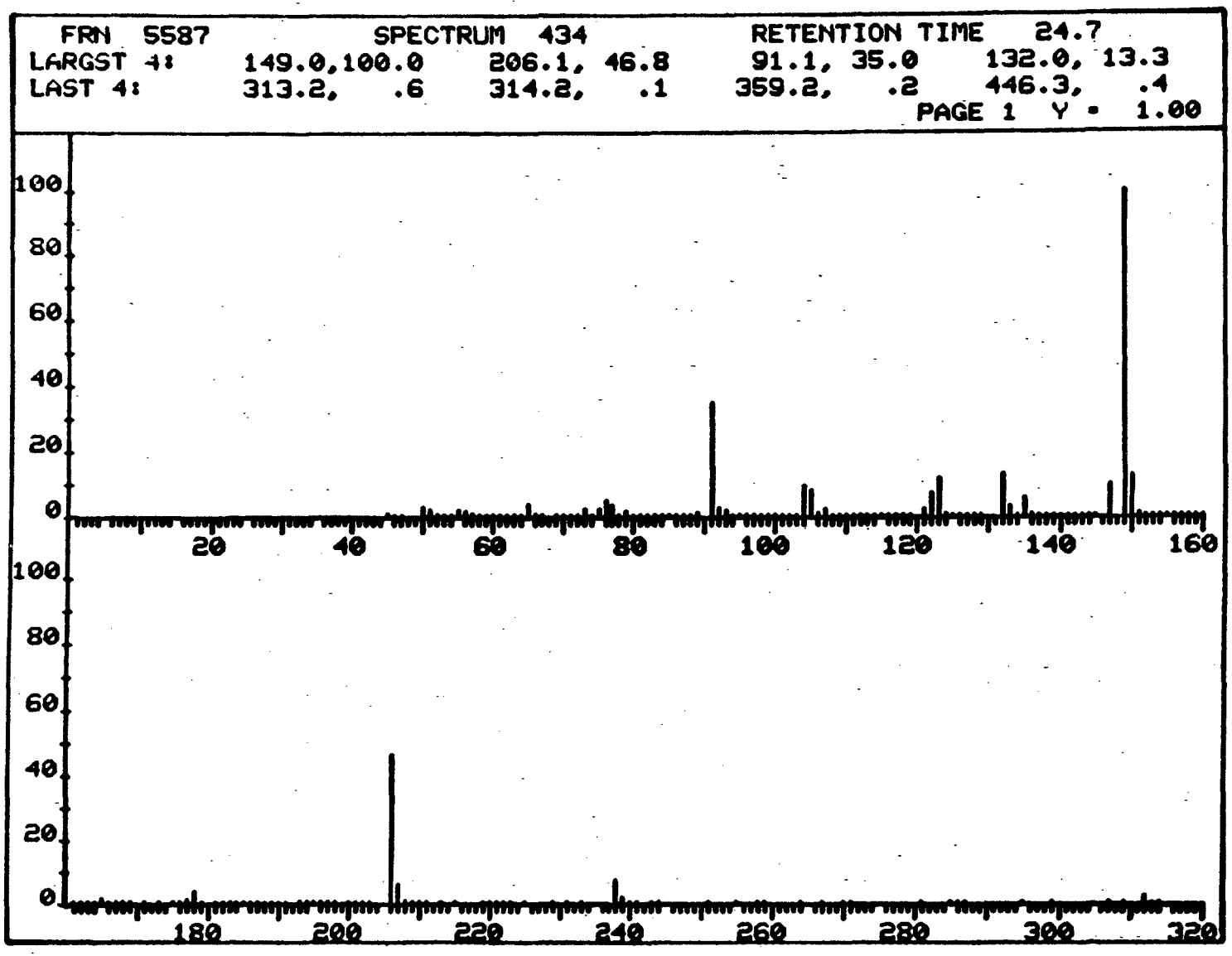
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5587
SPECTRUM 434
RETENTION TIME 24.7
LARGST 4: 149.0,100.0 206.1, 46.8 91.1, 35.0 132.0, 13.3
LAST 4: 313.2, .6 314.2, .1 359.2, .2 446.3, .4
PAGE 1 Y = 1.00
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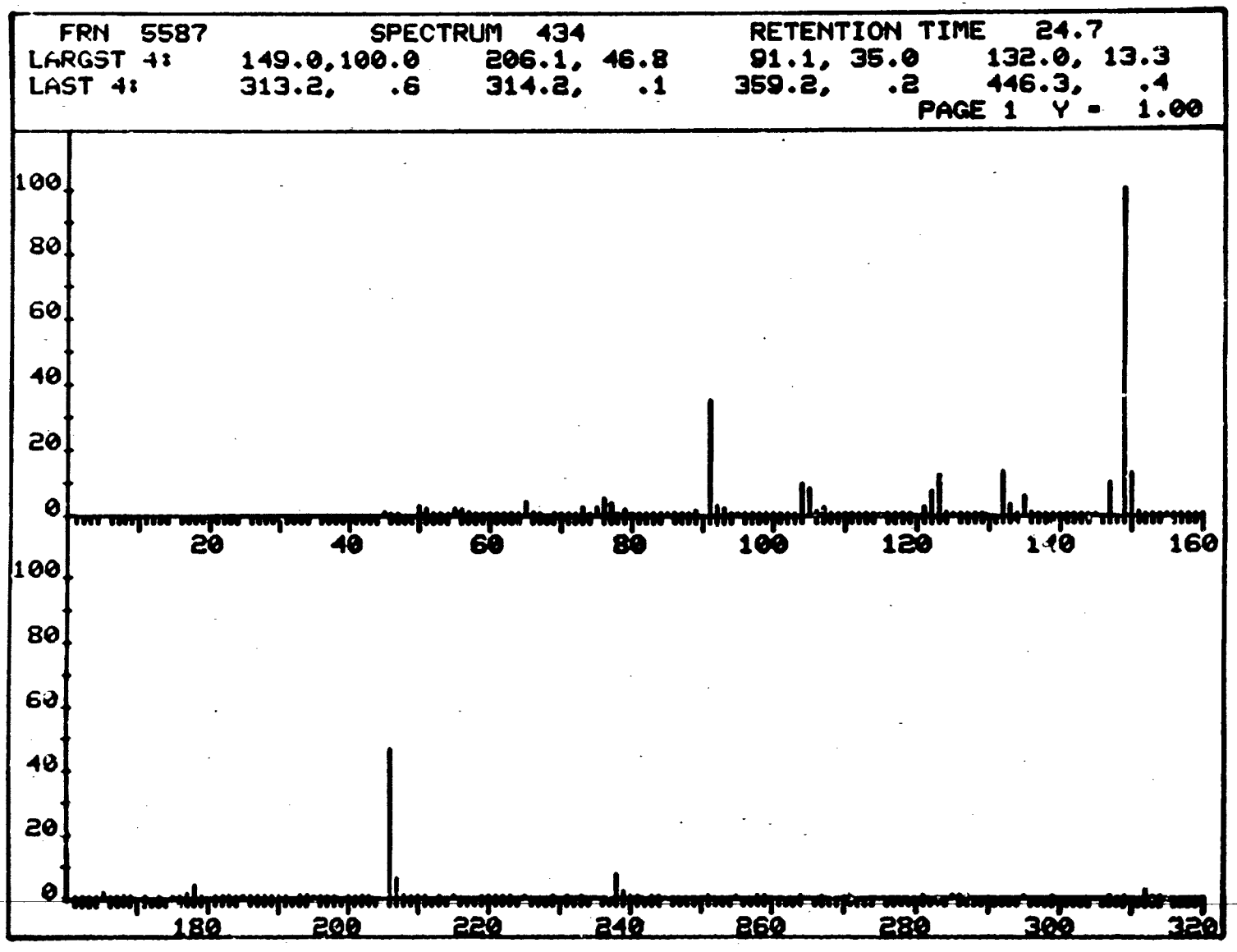
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5587
SPECTRUM 434
RETENTION TIME 24.7
LARGST 4: 149.0,100.0 206.1, 46.8 91.1, 35.0 132.0, 13.3
LAST 4: 313.2, .6 314.2, .1 359.2, .2 446.3, .4
PAGE 1 Y = 1.00
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40
20
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20 40 60 80 100 120 140 160
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80
60
40
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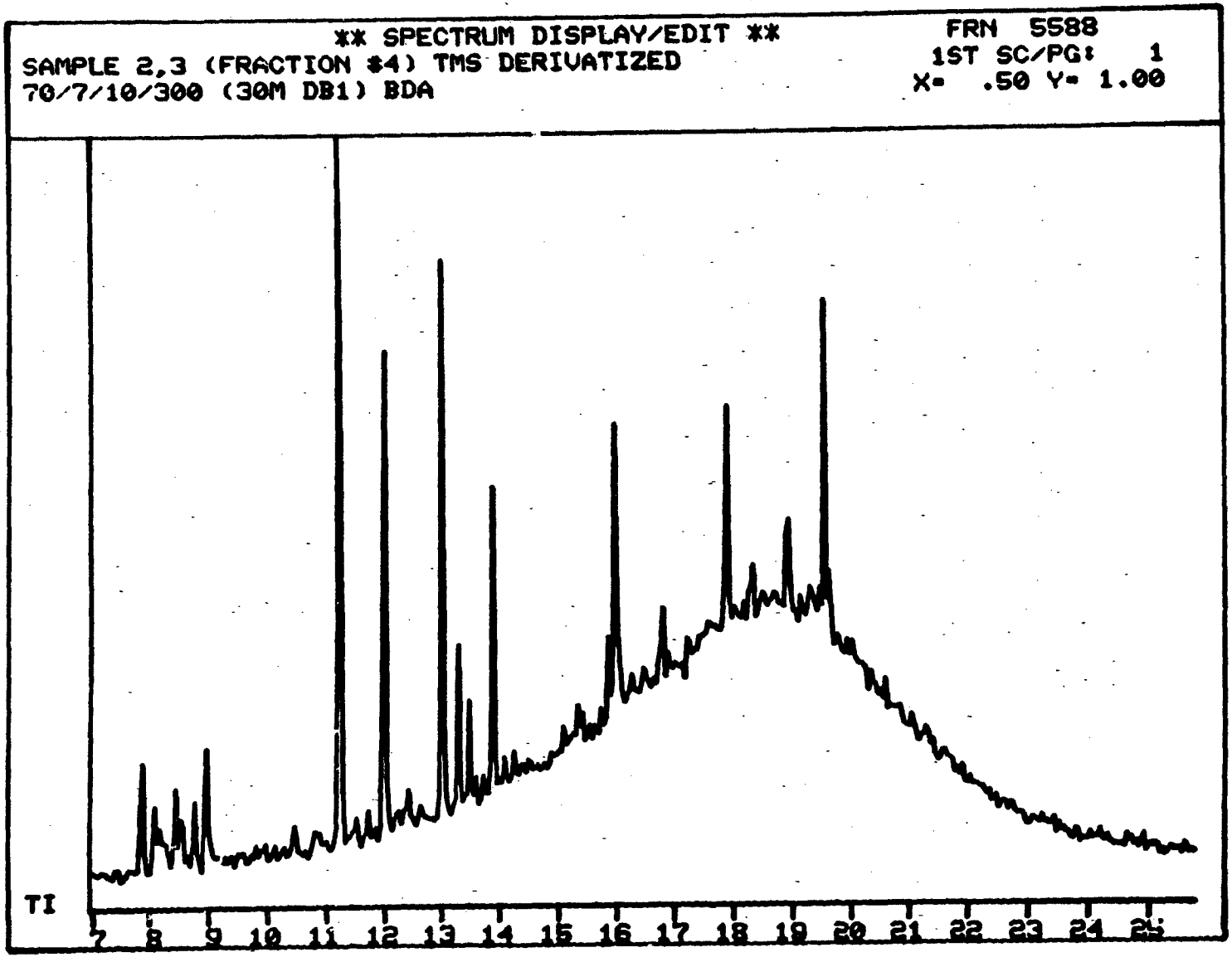
0
180 200 220 240 260 280 300 320


** SPECTRUM DISPLAY/EDIT **
FRN 5588
SAMPLE 2,3 (FRACTION #4) TMS DERIVATIZED
1ST SC/PG: 1
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X= .50 Y= 1.00
TI
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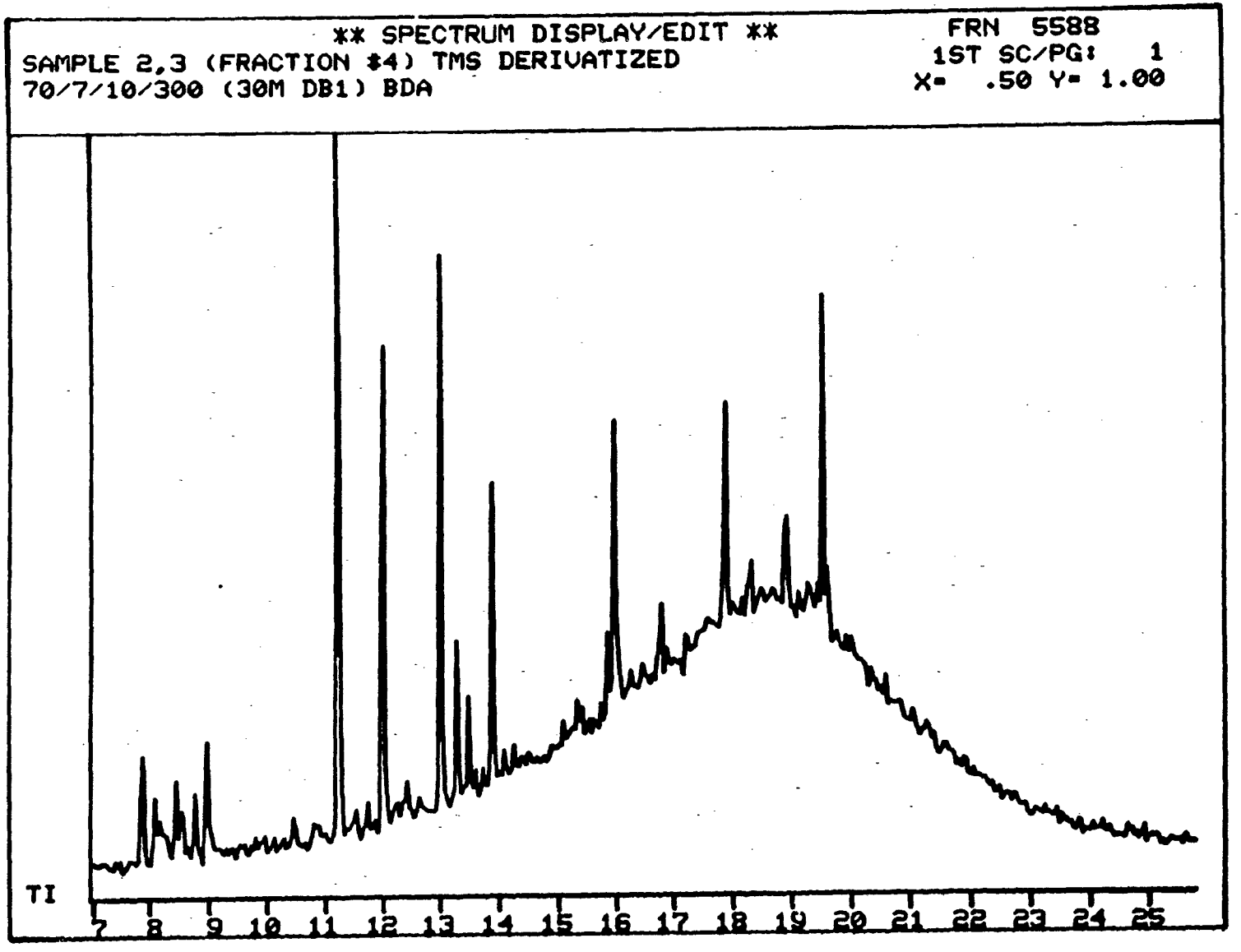
** SPECTRUM DISPLAY/EDIT **
FRN 5588
SAMPLE 2,3 (FRACTION #4) TMS DERIVATIZED
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70/7/10/300 (30M DB1) BDA
X= .50 Y= 1.00
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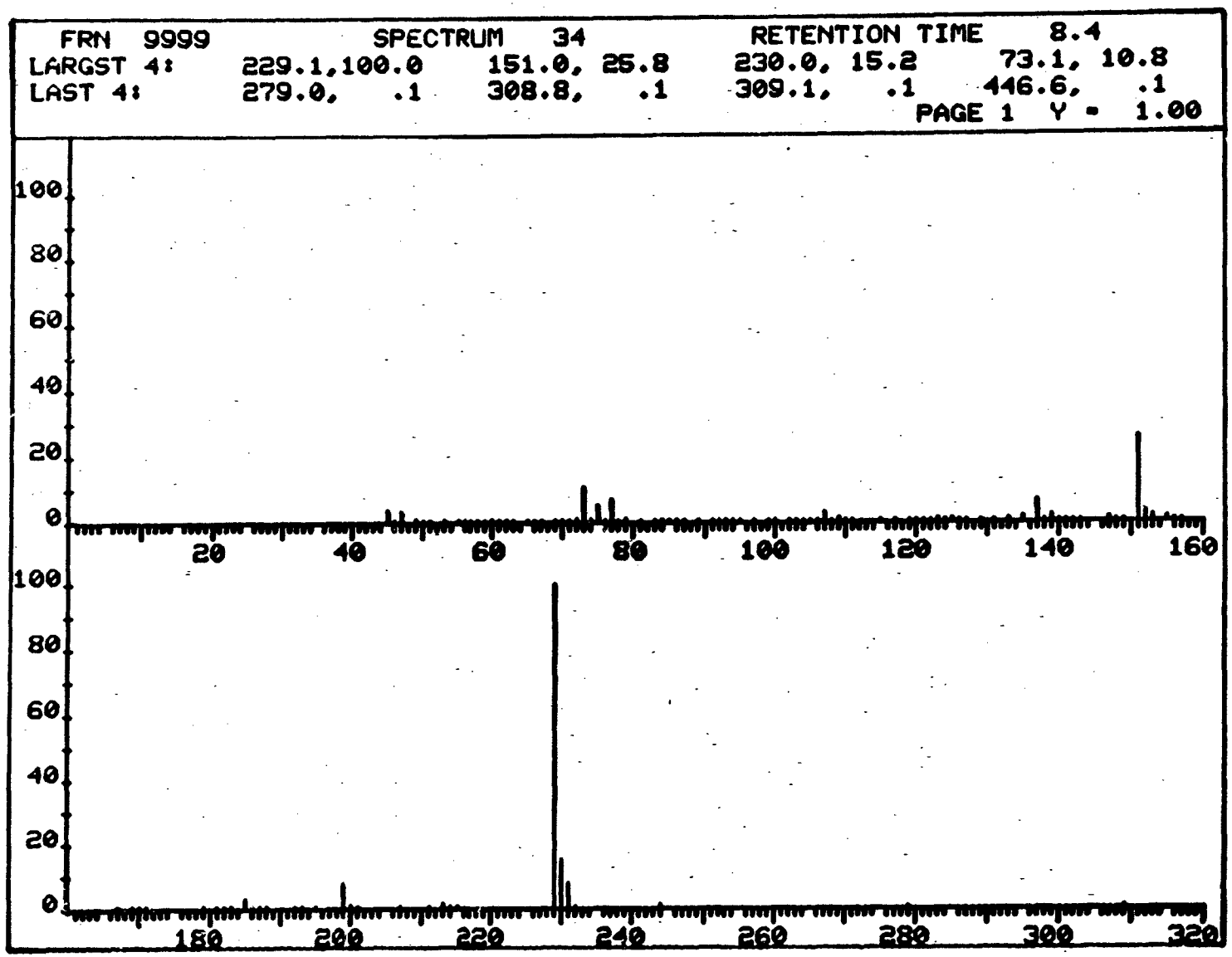
FRN 9999
SPECTRUM 34
RETENTION TIME 8.4
LARGST 4: 229.1, 100.0 151.0, 25.8 230.0, 15.2 73.1, 10.8
LAST 4: 279.0, .1 308.8, .1 309.1, .1 446.6, .1
PAGE 1 Y = 1.00
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40
20
0
20 40 60 80 100 120 140 160
100
80
60
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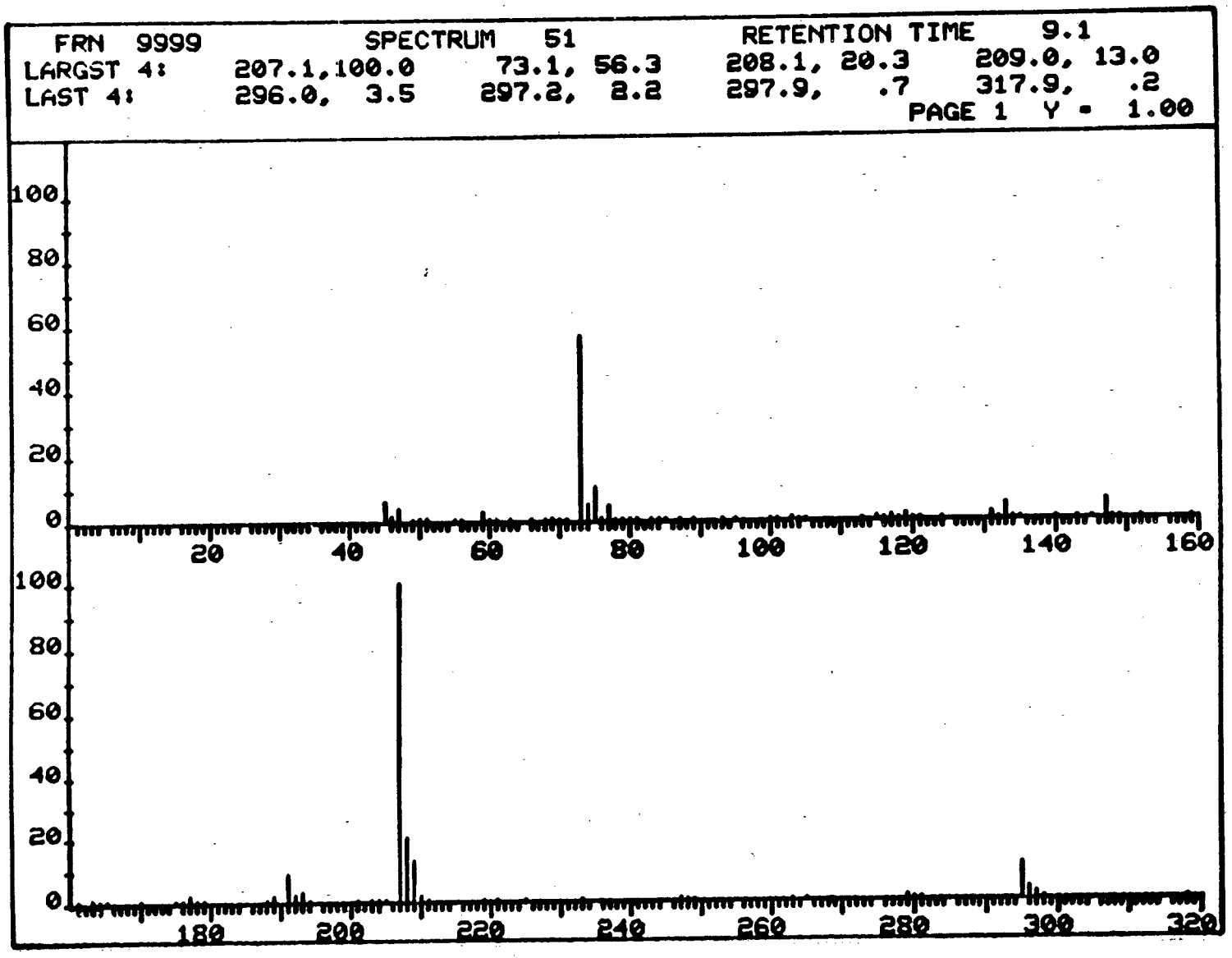
0
180 200 220 240 260 280 300 320


FRN 9999
SPECTRUM 51
RETENTION TIME 9.1
LARGST 4: 207.1,100.0 73.1, 56.3 208.1, 20.3 209.0, 13.0
LAST 4: 296.0, 3.5 297.2, 2.2 297.9, .7 317.9, .2
PAGE 1 Y = 1.00
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40
20
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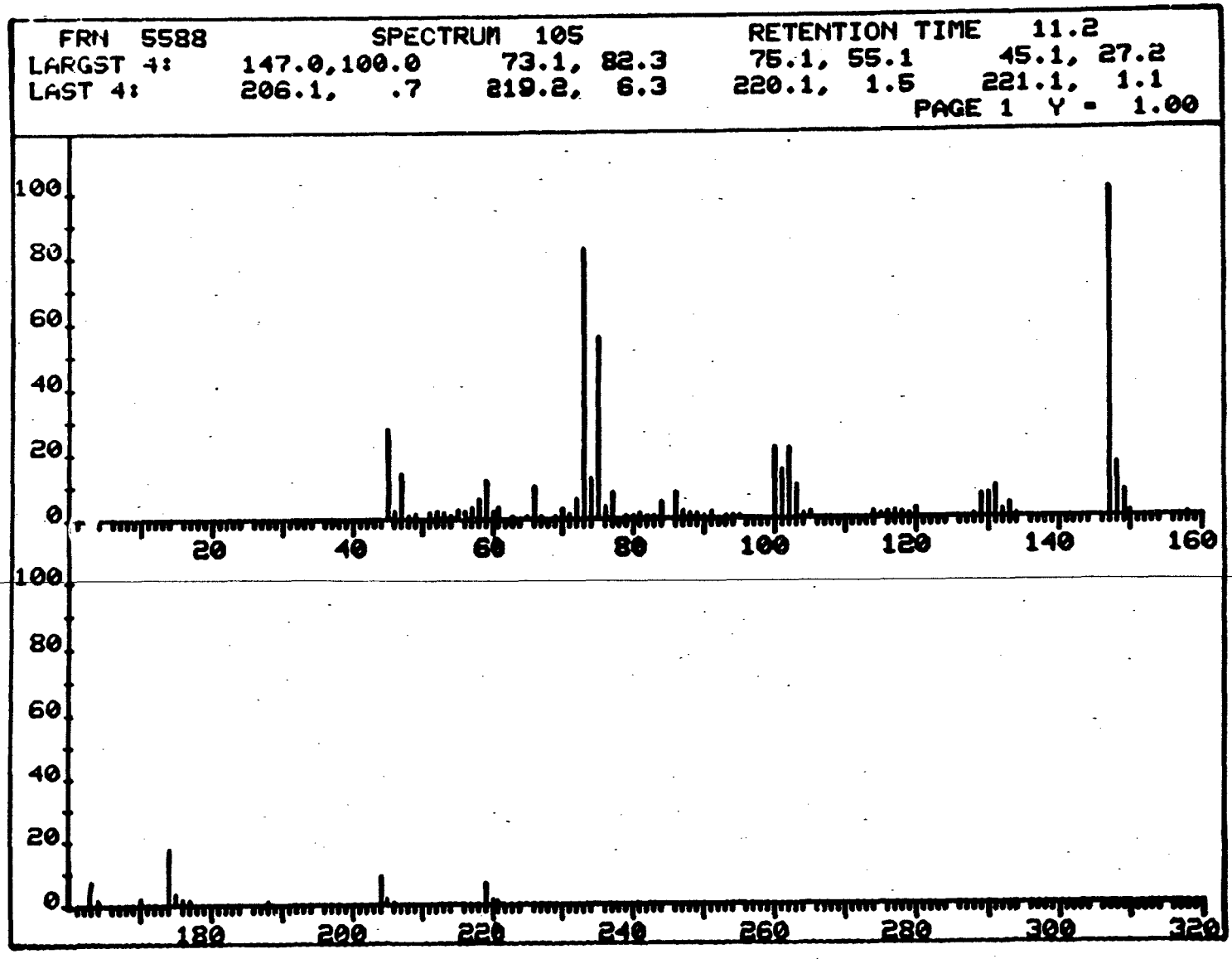
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5588
SPECTRUM 105
RETENTION TIME 11.2
LARGST 4: 147.0,100.0 73.1, 82.3 75.1, 55.1 45.1, 27.2
LAST 4: 206.1, .7 219.2, 6.3 220.1, 1.5 221.1, 1.1
PAGE 1 Y = 1.00
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300
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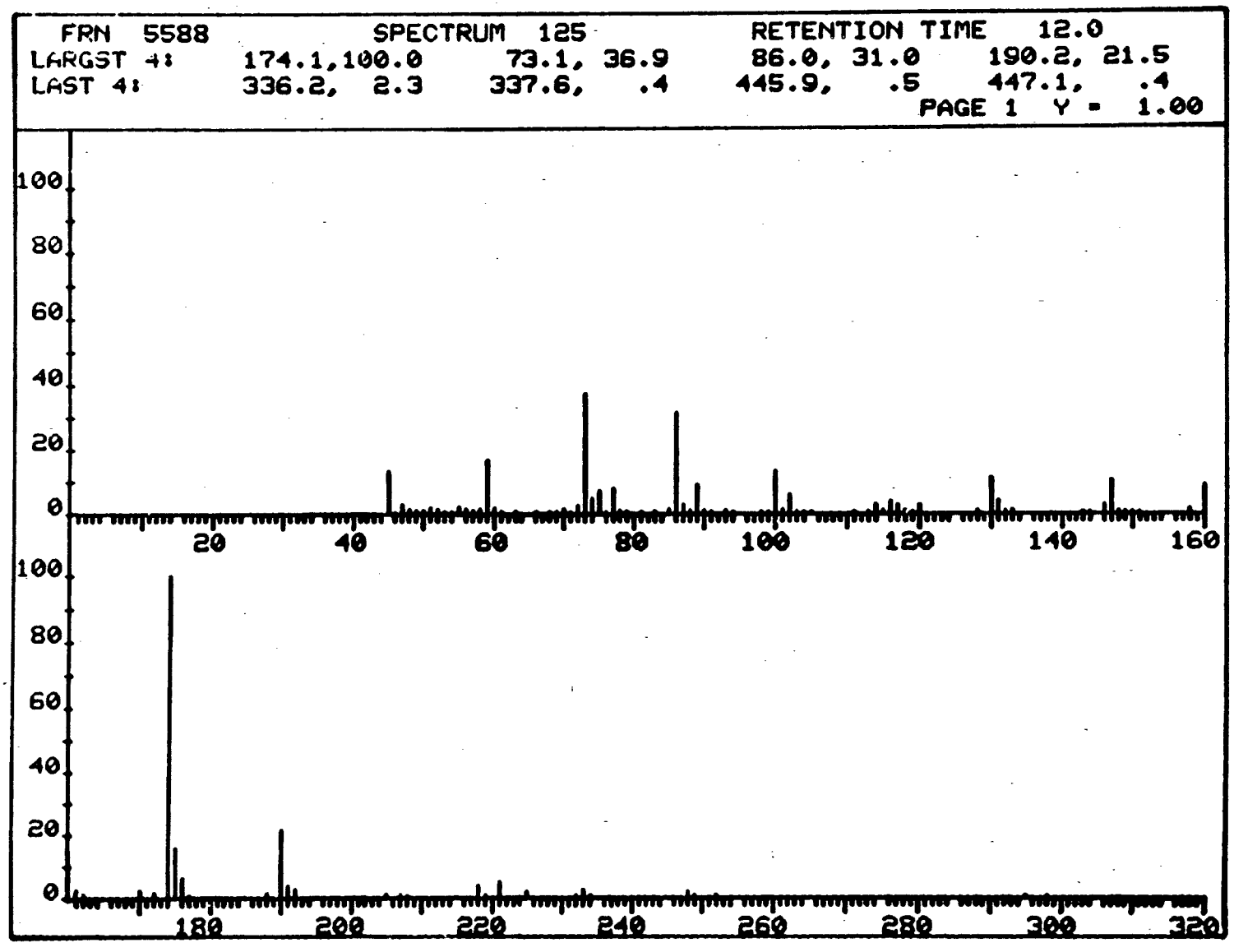
FRN 5588
SPECTRUM 125
RETENTION TIME 12.0
LARGST 4: 174.1,100.0 73.1, 36.9 86.0, 31.0 190.2, 21.5
LAST 4: 336.2, 2.3 337.6, .4 445.9, .5 447.1, .4
PAGE 1 Y = 1.00
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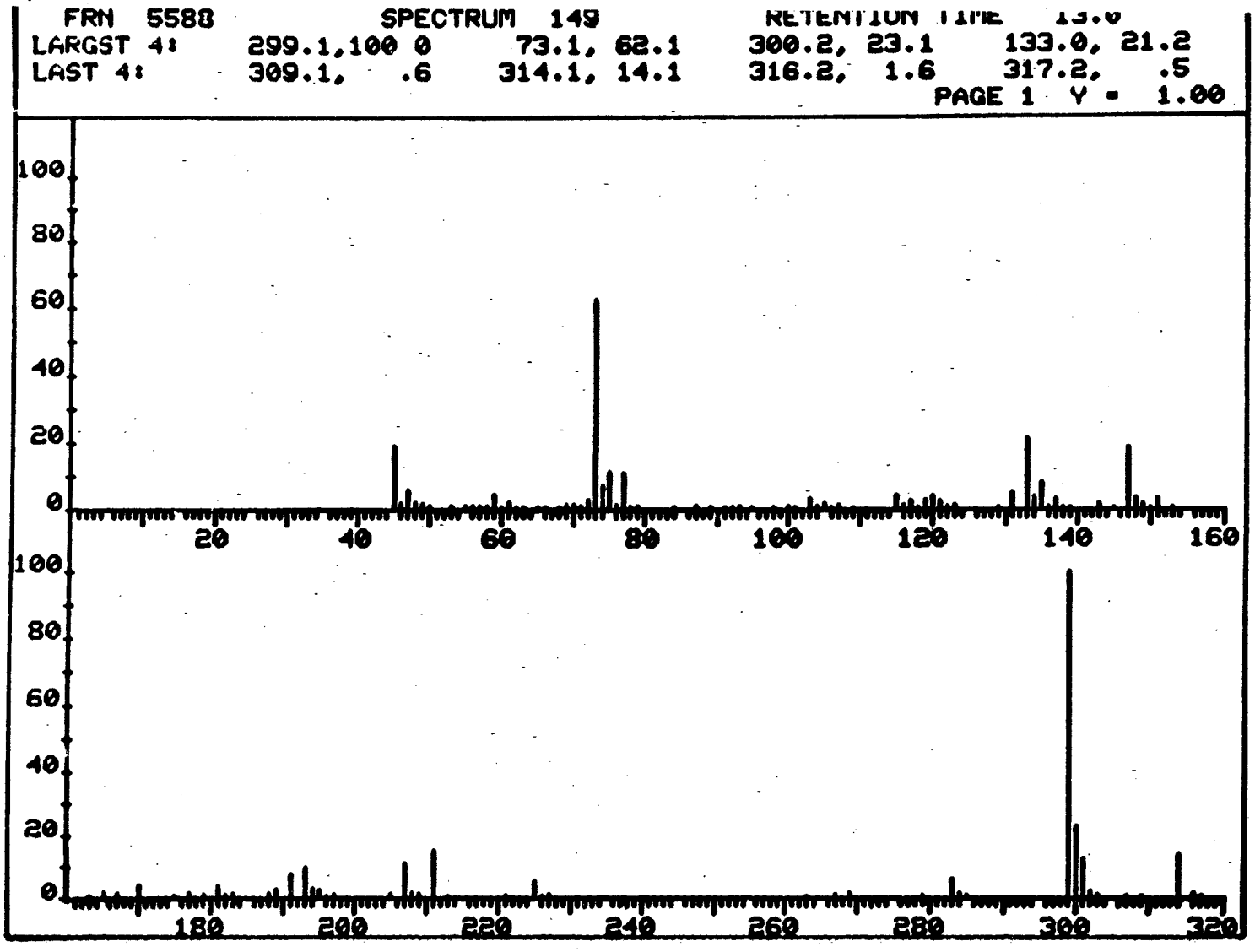
60
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5588
SPECTRUM 149
RETENTION TIME
LARGST 4: 299.1,100 0 73.1, 62.1 300.2, 23.1 133.0, 21.2
LAST 4: 309.1, .6 314.1, 14.1 316.2, 1.6 317.2, .5
PAGE 1 Y = 1.00
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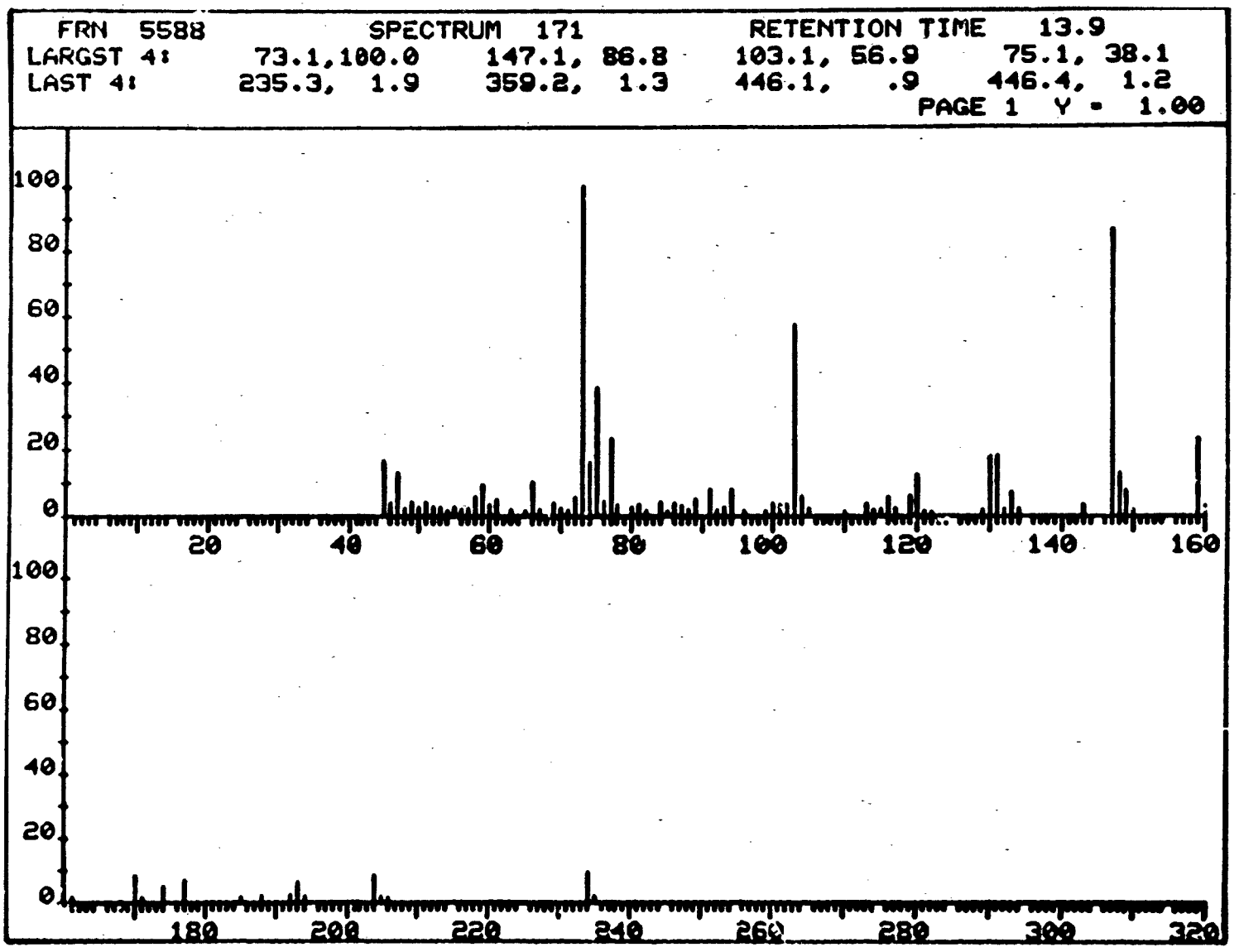
60
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5588
SPECTRUM 171
RETENTION TIME 13.9
LARGST 4: 73.1,100.0 147.1, 86.8 103.1, 56.9 75.1, 38.1
LAST 4: 235.3, 1.9 359.2, 1.3 446.1, .9 446.4, 1.2
PAGE 1 Y = 1.00
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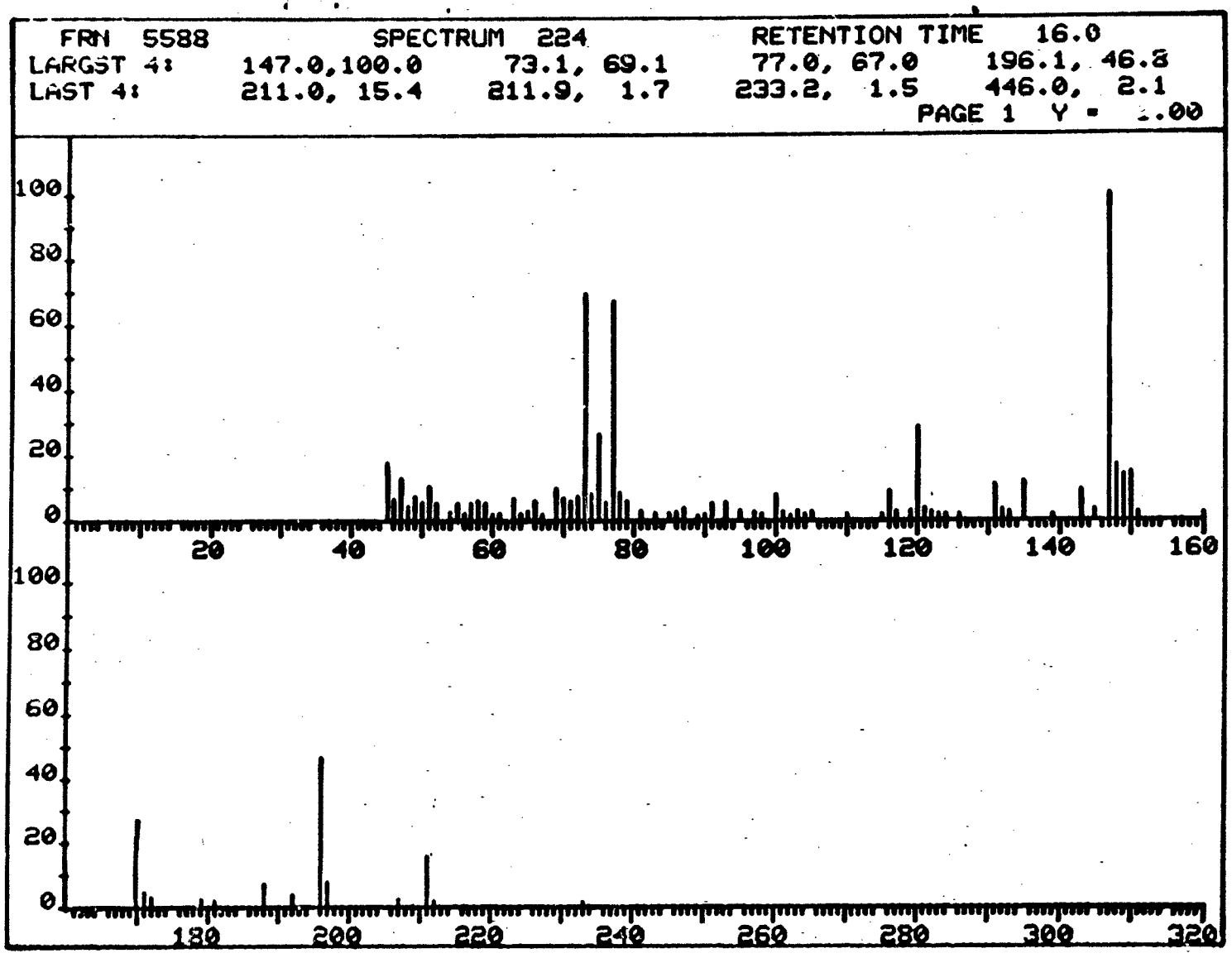
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320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SPECTRUM 224
RETENTION TIME 16.0
LARGST 4: 147.0,100.0 73.1, 69.1 77.0, 67.0 196.1, 46.8
LAST 4: 211.0, 15.4 211.9, 1.7 233.2, 1.5 446.0, 2.1
PAGE 1 Y = 1.00
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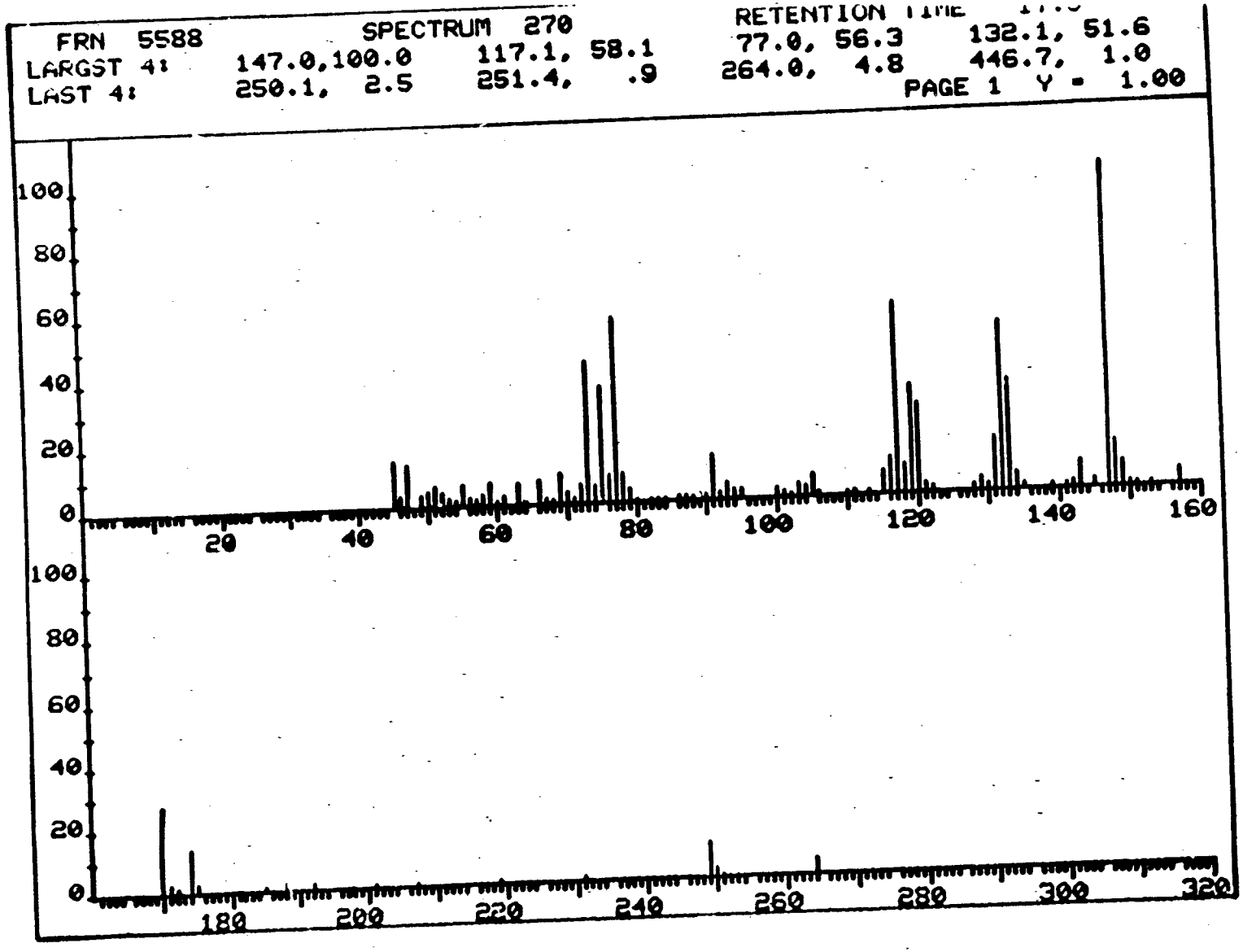
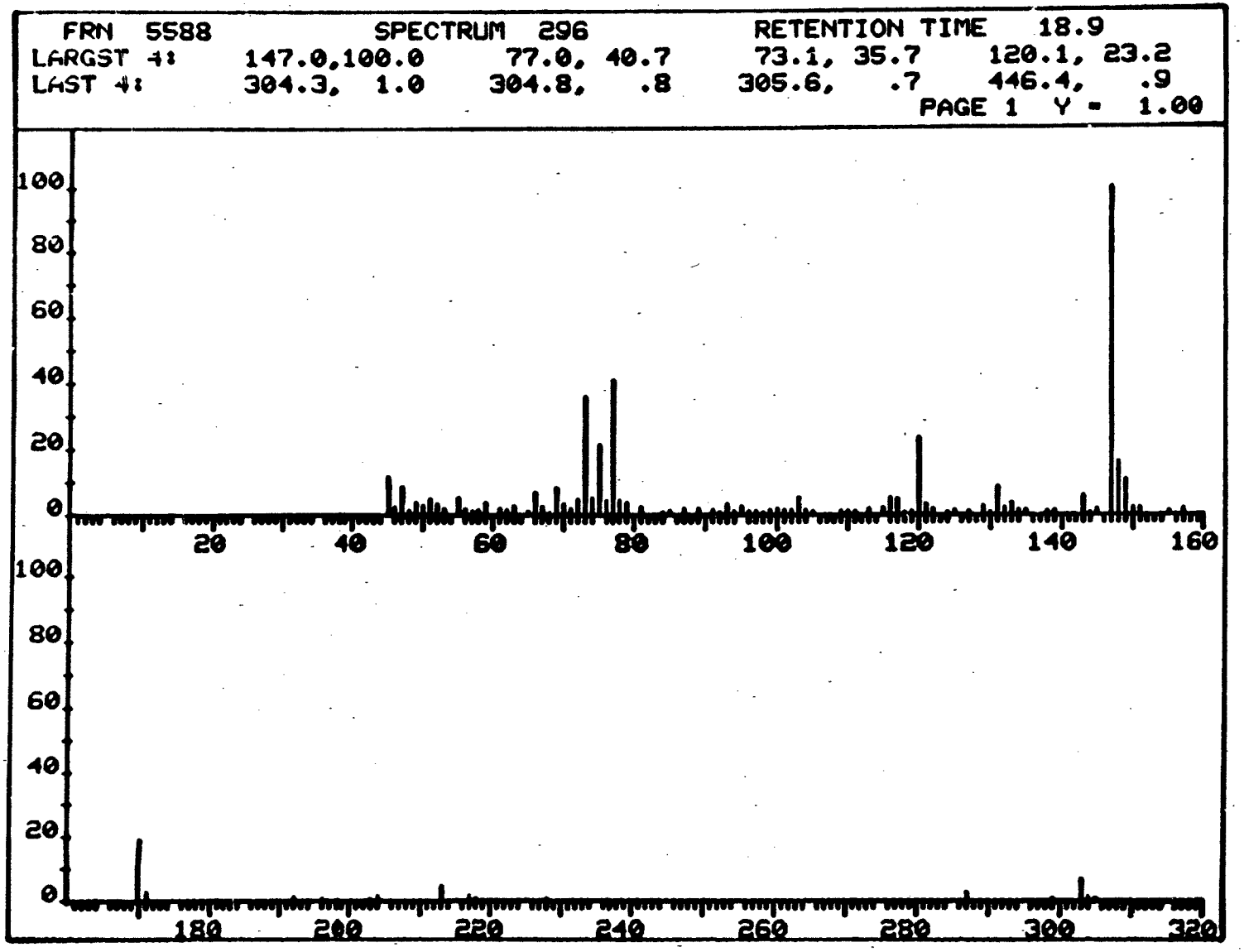
60
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


FRN 5588
SPECTRUM 270
RETENTION TIME
LARGST 4: 147.0,100.0 117.1, 58.1 77.0, 56.3 132.1, 51.6
LAST 4: 250.1, 2.5 251.4, .9 264.0, 4.8 446.7, 1.0
PAGE 1 Y = 1.00
100
80
60
40
20
0
20
40
60
80
100
120
140
160
100
80
60
40
20
0
180
200
220
240
260
280
300
320


FRN 5588
SPECTRUM 296
RETENTION TIME 18.9
LARGST 4: 147.0,100.0 77.0, 40.7 73.1, 35.7 120.1, 23.2
LAST 4: 304.3, 1.0 304.8, .8 305.6, .7 446.4, .9
PAGE 1 Y = 1.00
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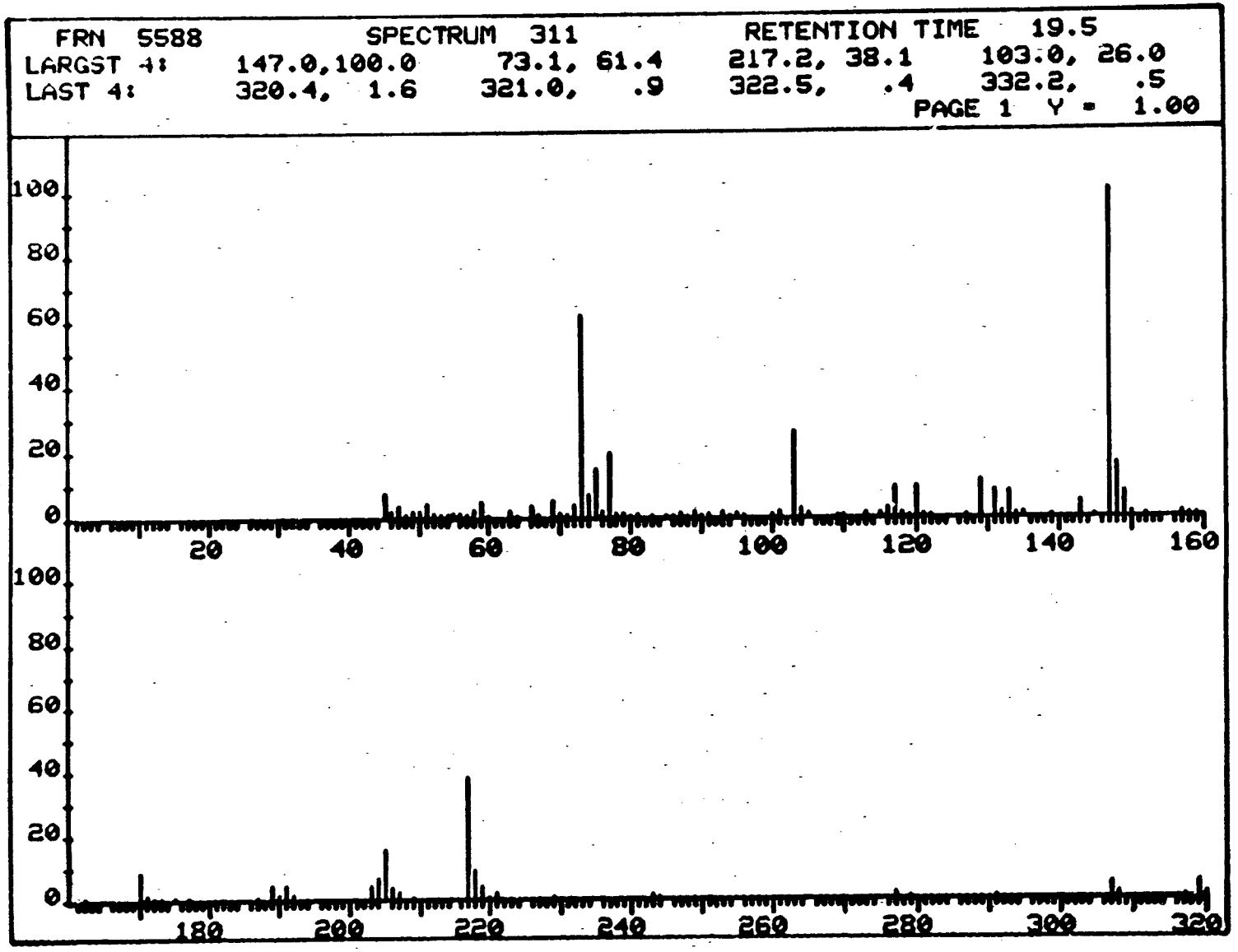
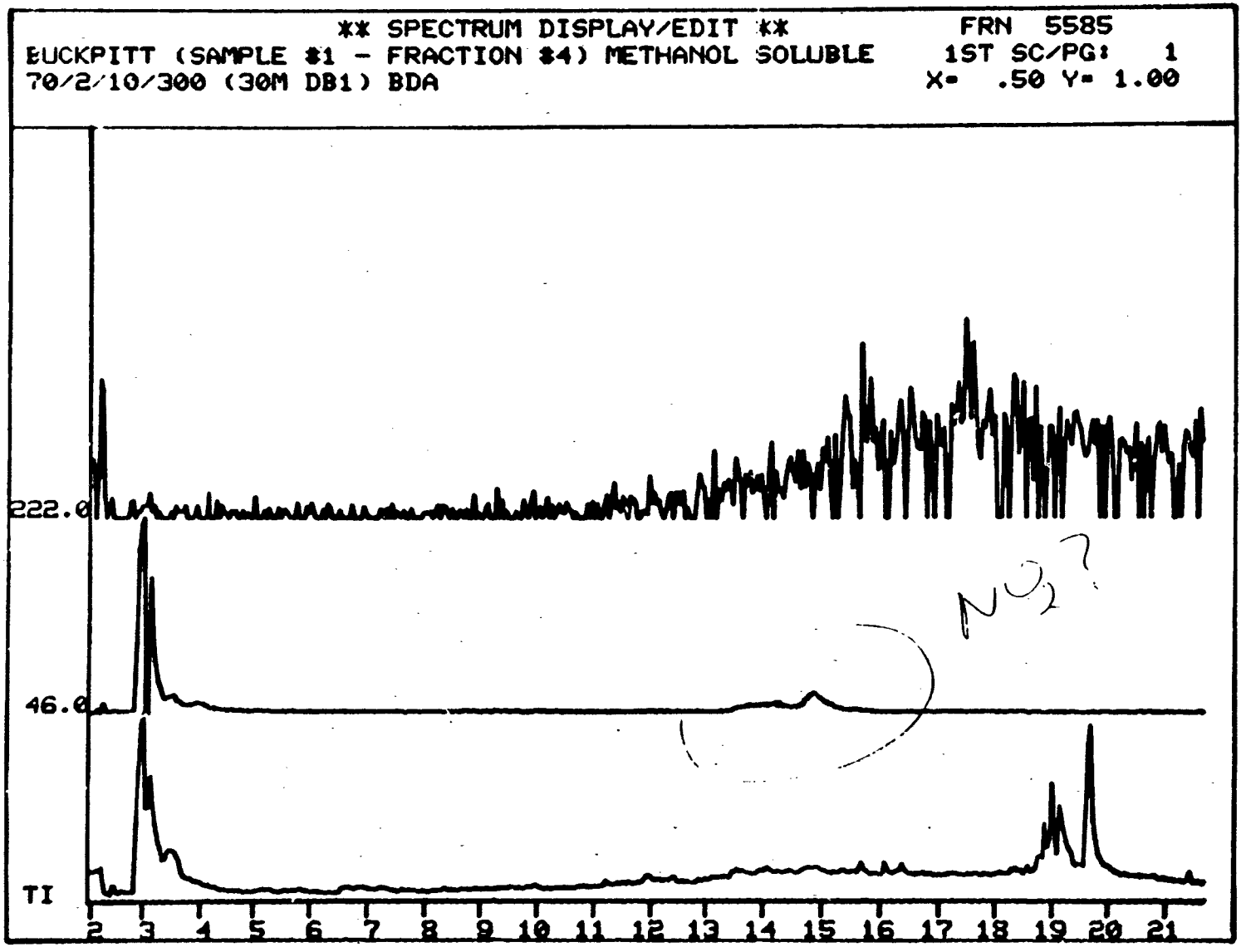
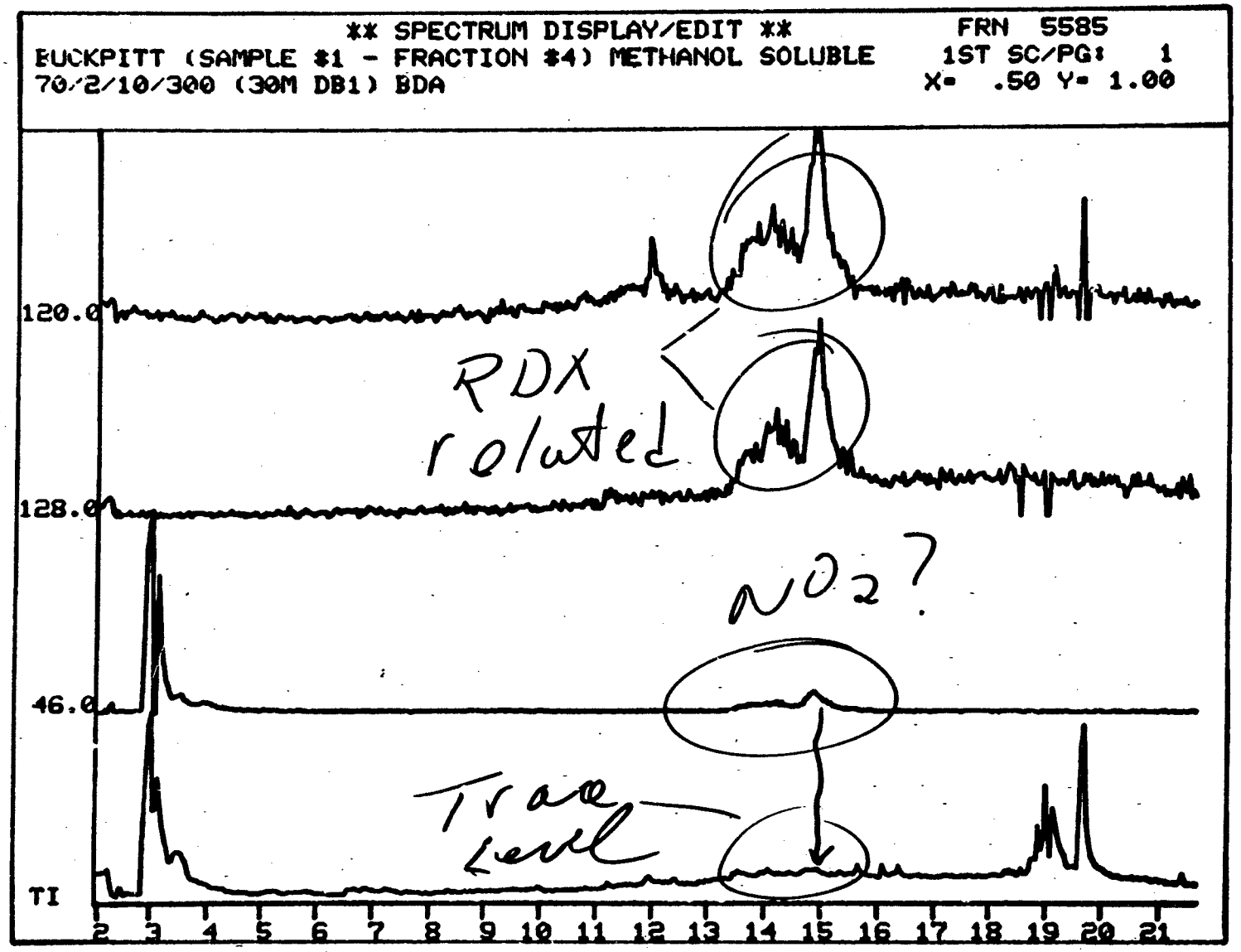
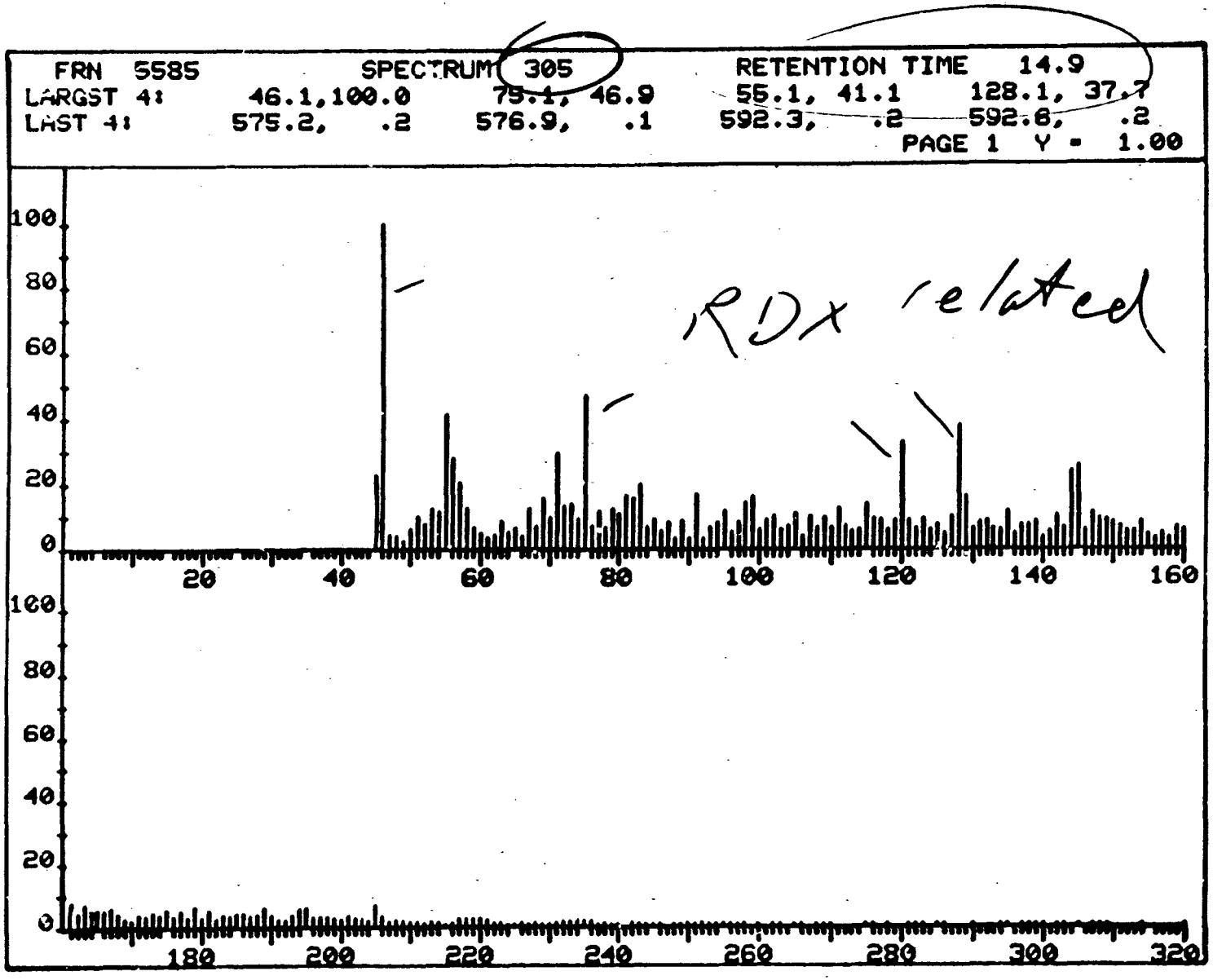
80
60
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320

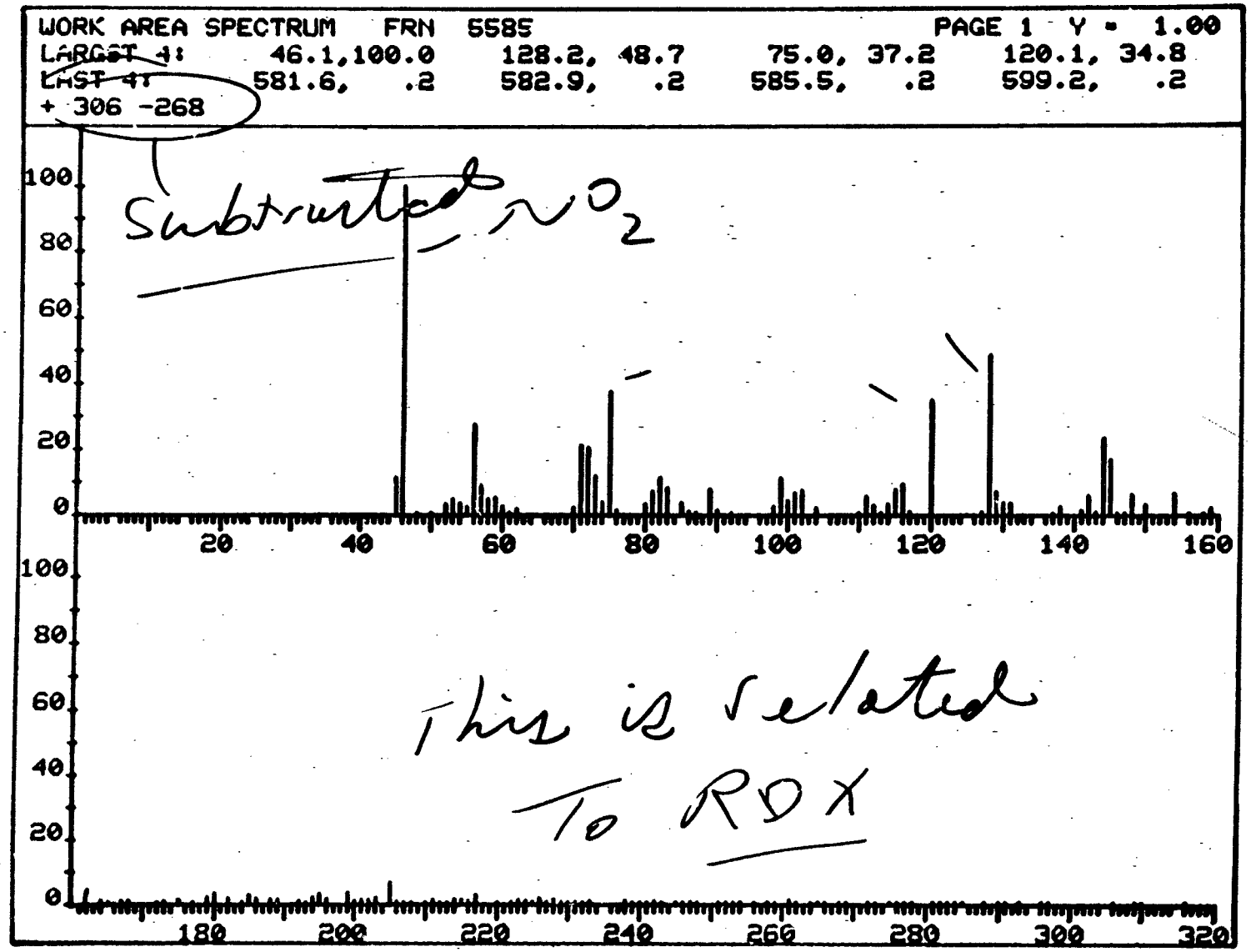

FRN 5588
SPECTRUM 311
RETENTION TIME 19.5
LARGST 4: 147.0, 100.0 73.1, 61.4 217.2, 38.1 103.0, 26.0
LAST 4: 320.4, 1.6 321.0, .9 322.5, .4 332.2, .5
PAGE 1 Y = 1.00
100
80
60
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320

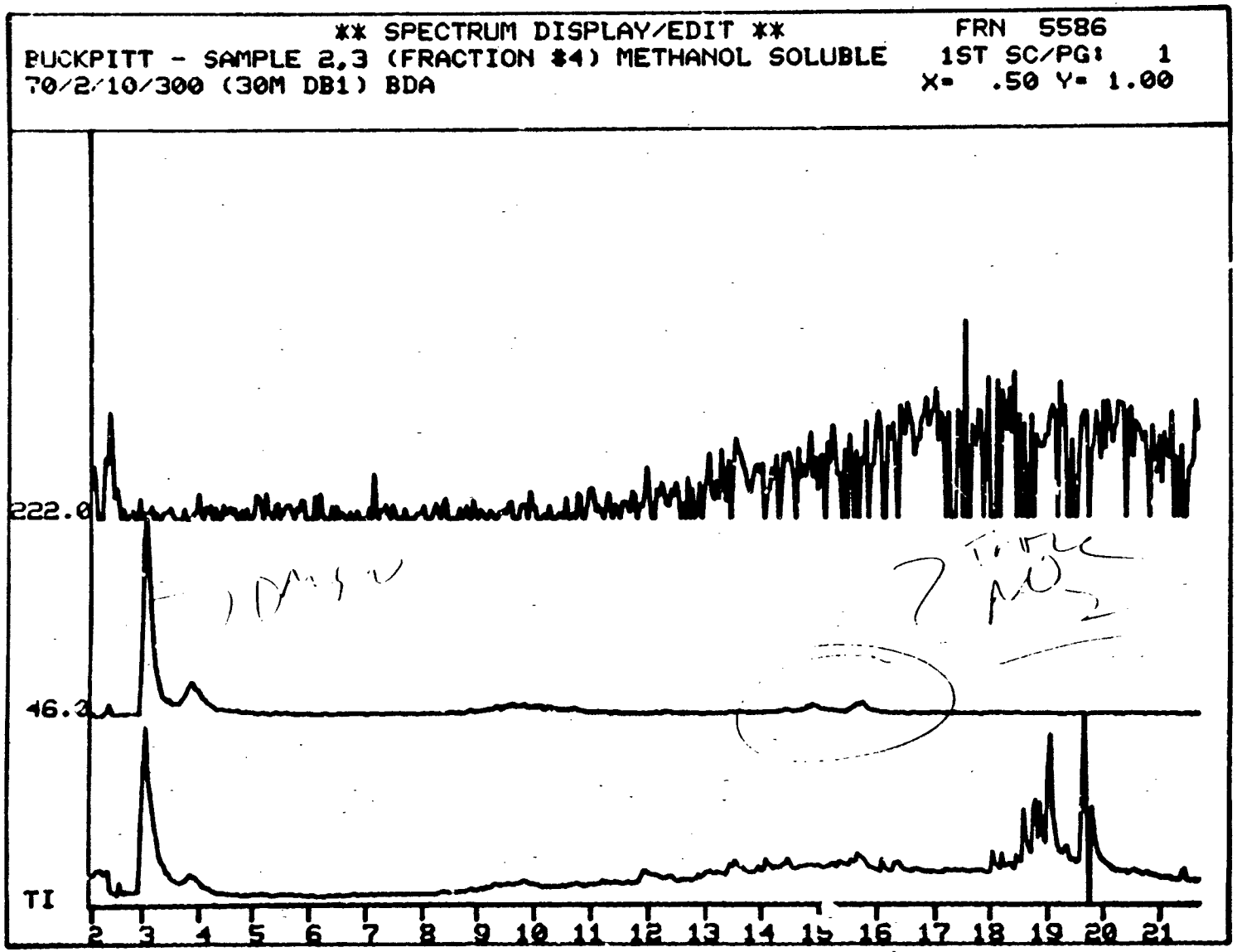
Mass Chromatogram Plots of Selected Classes of Compounds


** SPECTRUM DISPLAY/EDIT **
FRN 5585
EUCKPITT (SAMPLE #1 - FRACTION #4) METHANOL SOLUBLE
1ST SC/PG: 1
70/2/10/300 (30M DB1) BDA
X= .50 Y= 1.00
222.0
46.0
TI
NO2?
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


** SPECTRUM DISPLAY/EDIT **
FRN 5585
BUCKPITT (SAMPLE #1 - FRACTION #4) METHANOL SOLUBLE
1ST SC/PG: 1
70/2/10/300 (30M DB1) BDA
X= .50 Y= 1.00
120.0
RDX
related
128.0
NO2?
46.0
Trace
level
TI
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


FRN 5585
SPECTRUM 305
RETENTION TIME 14.9
LARGST 4: 46.1,100.0 79.1, 46.9 55.1, 41.1 128.1, 37.7
LAST 4: 575.2, .2 576.9, .1 592.3, .2 592.6, .2
PAGE 1 Y = 1.00
RDX related
100
80
60
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


WORK AREA SPECTRUM FRN 5585 PAGE 1 Y = 1.00
LARGST 4: 46.1,100.0 128.2, 48.7 75.0, 37.2 120.1, 34.8
LAST 4: 581.6, .2 582.9, .2 585.5, .2 599.2, .2
+ 306 -268
Subtracted NO2
This is related To RDX
100
80
60
40
20
0
20 40 60 80 100 120 140 160
100
80
60
40
20
0
180 200 220 240 260 280 300 320


** SPECTRUM DISPLAY/EDIT **
FRN 5586
BUCKPITT - SAMPLE 2,3 (FRACTION #4) METHANOL SOLUBLE
1ST SC/PG: 1
70/2/10/300 (30M DB1) BDA
X= .50 Y= 1.00
222.0
46.3
TI
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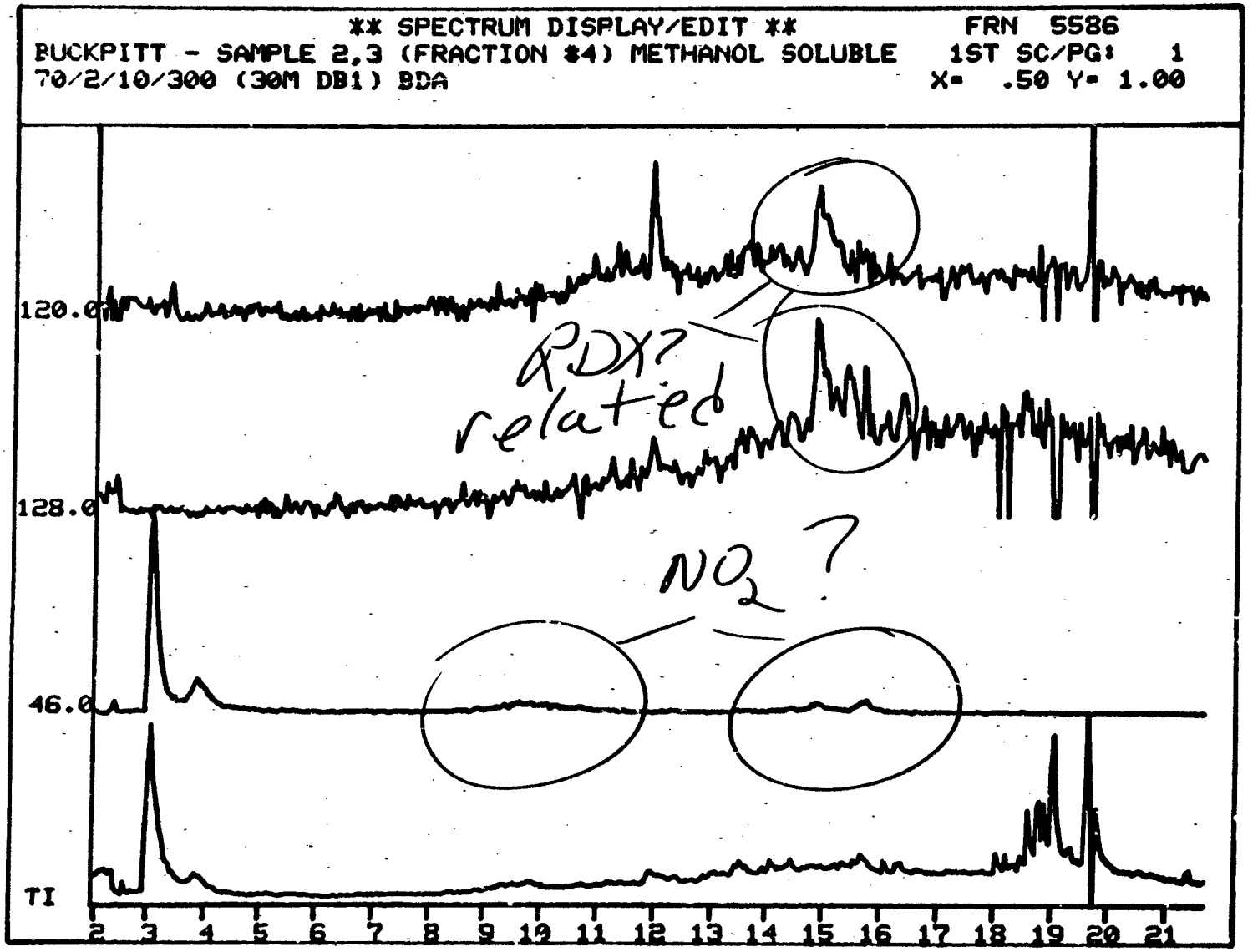
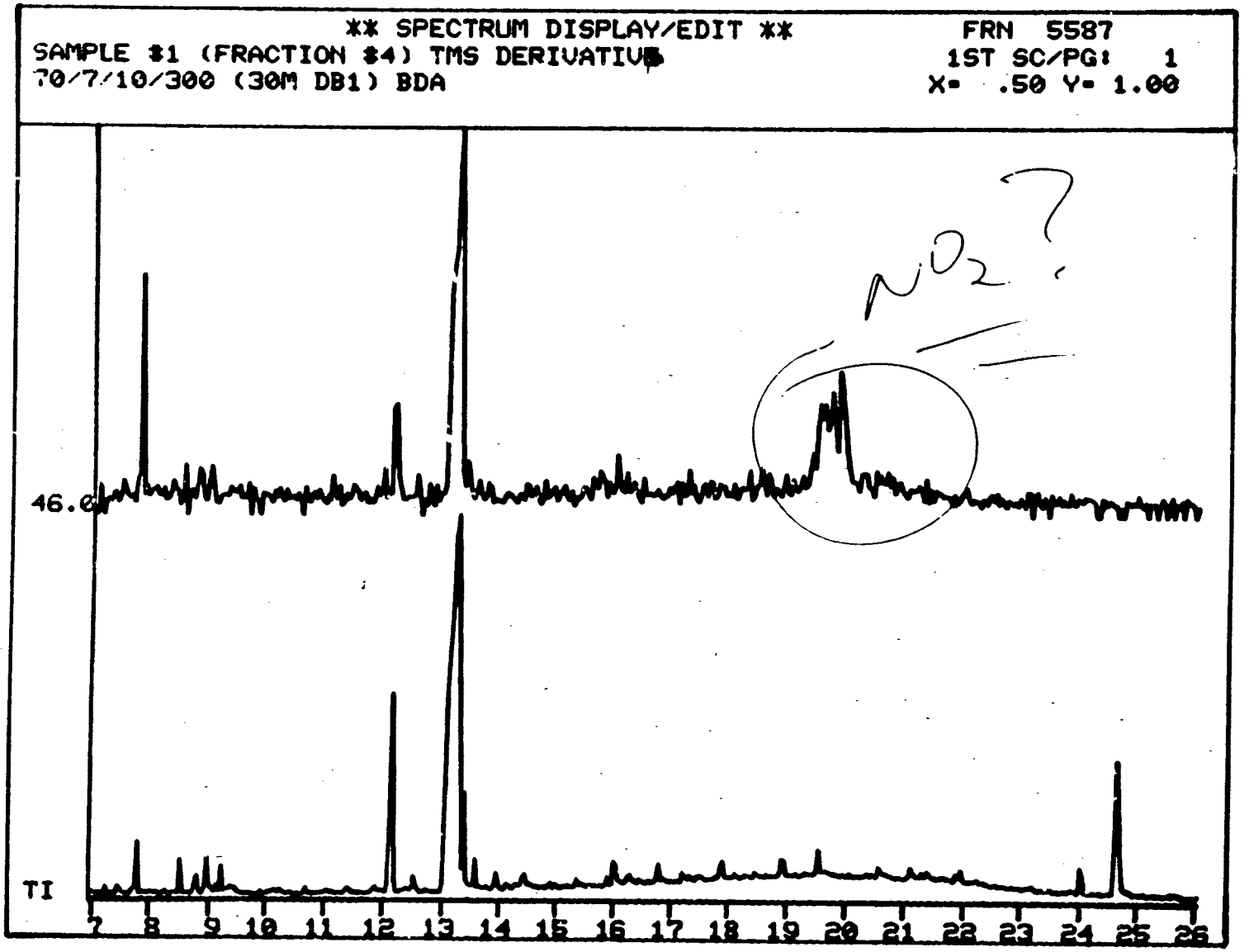
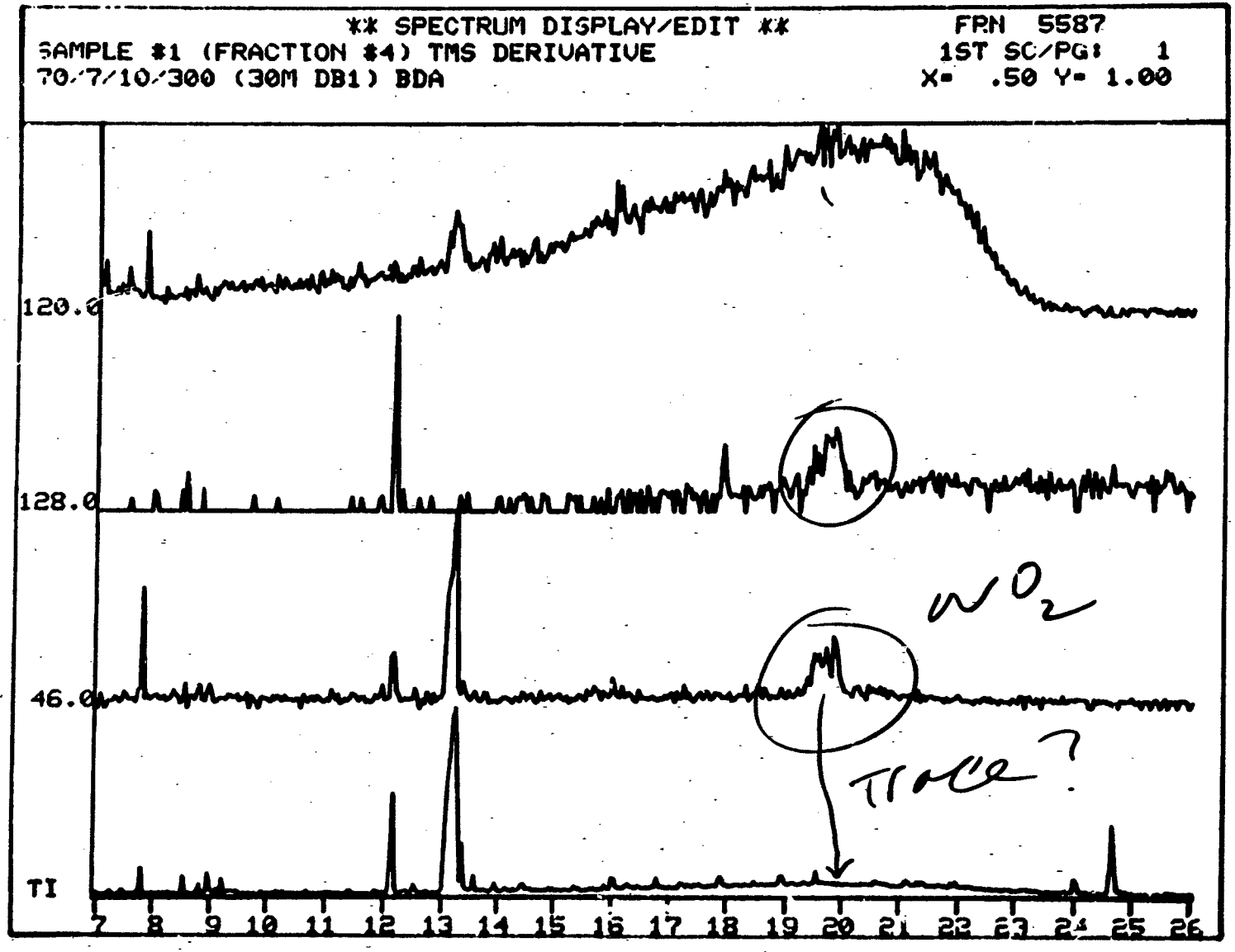
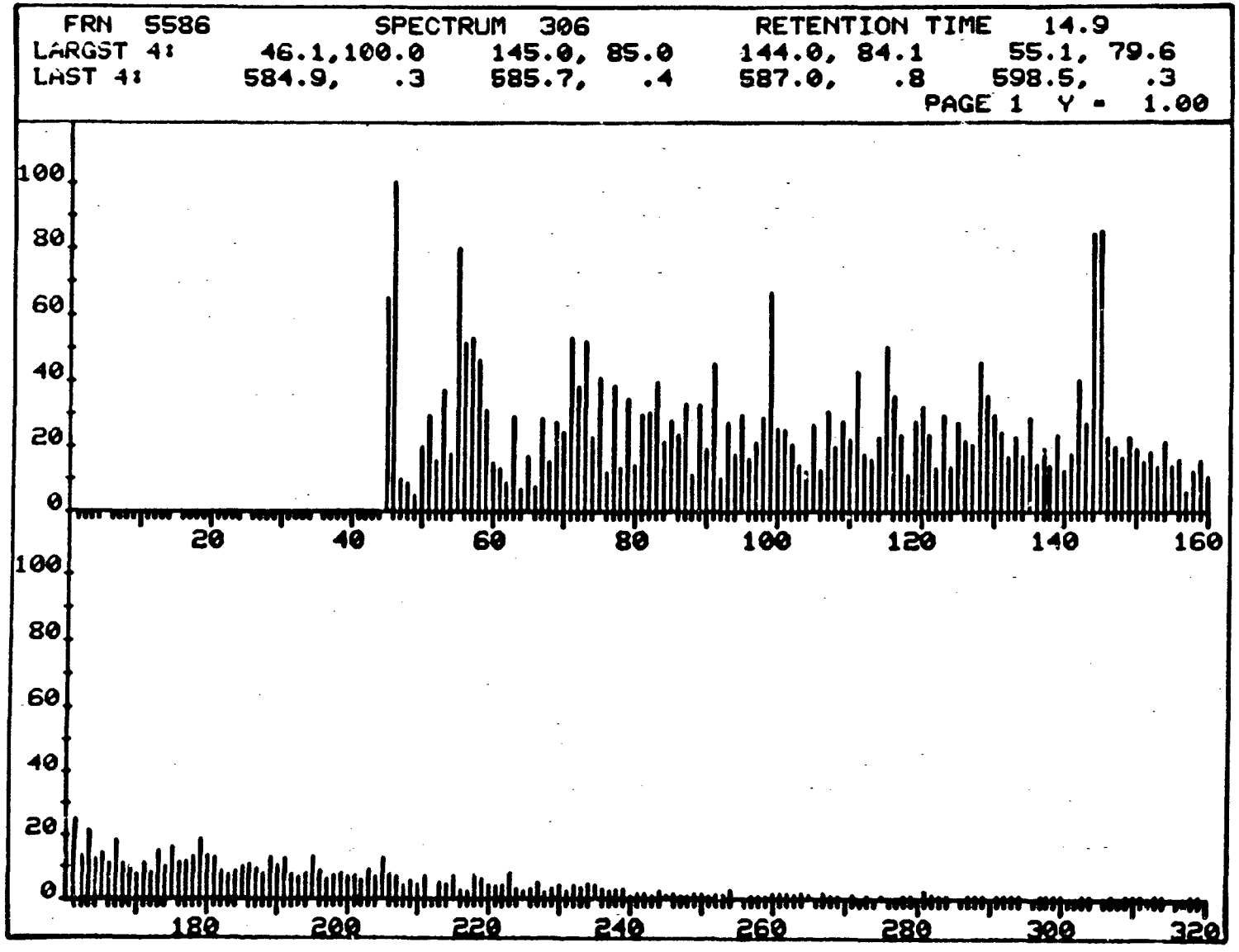

** SPECTRUM DISPLAY/EDIT **
FRN 5586
BUCKPITT - SAMPLE 2,3 (FRACTION #4) METHANOL SOLUBLE
1ST SC/PG: 1
70/2/10/300 (30M DB1) BDA
X= .50 Y= 1.00
120.0
128.0
46.0
TI
RDX?
related
NO2 ?
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


** SPECTRUM DISPLAY/EDIT **
FRN 5587
SAMPLE #1 (FRACTION #4) TMS DERIVATIVE
1ST SC/PG: 1
70/7/10/300 (30M DB1) BDA
X= .50 Y= 1.00
NO2 ?
46.0
TI
2 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26


** SPECTRUM DISPLAY/EDIT **
FRN 5587
SAMPLE #1 (FRACTION #4) TMS DERIVATIVE
1ST SC/PG: 1
70/7/10/300 (30M DB1) BDA
X= .50 Y= 1.00
120.0
128.0
46.0
TI
NO2
Trace?
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26


FRN 5586
SPECTRUM 306
RETENTION TIME 14.9
LARGST 4: 46.1,100.0 145.0, 85.0 144.0, 84.1 55.1, 79.6
LAST 4: 584.9, .3 585.7, .4 587.0, .8 598.5, .3
PAGE 1 Y = 1.00
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0
20
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260
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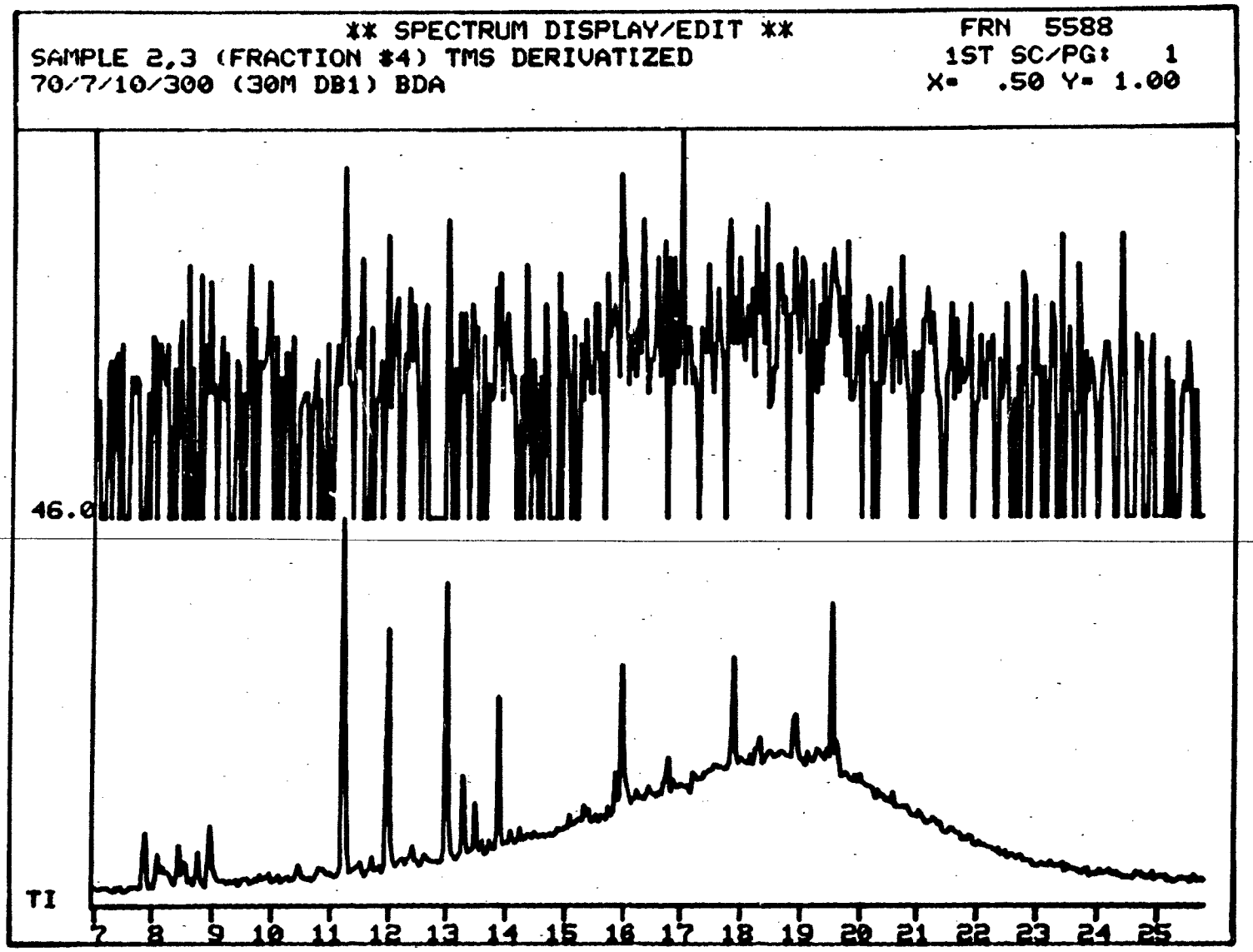
300
320


** SPECTRUM DISPLAY/EDIT **
FRN 5588
SAMPLE 2,3 (FRACTION #4) TMS DERIVATIZED
1ST SC/PG: 1
70/7/10/300 (30M DB1) BDA
X= .50 Y= 1.00
46.0
TI
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Supporting Data

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SCHOOL OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY PHARMACOLOGY
AND TOXICOLOGY

DAVIS, CALIFORNIA 95616

April 20, 1987

Brian Andrasen, Ph.D.
Mail Drop L 453
P.O. Box 5507
Lawrence Livermore National Laboratory
Livermore, CA. 94550

Dear Brian:

I have enclosed two samples of "fraction 4" which contains the weak mutagen(s) that we have been attempting to identify. Several years ago the army considered altering the recrystallization solvent used in munitions manufacture from acetone/-cyclohexanone to DMSO. Due to the ability of DMSO to markedly enhance the absorption of xenobiotics through the skin, the sludge remaining after recrystallization was tested in the Ames assay for the possible presence of mutagens. As luck would have it, the material tested positive. We proposed to separate the mutagen by HPLC using the Ames assay as the monitoring tool and then to identify the material using mass spectrometry and proton NMR. One of the biggest problems with this project had been the very weak mutagenic activity of the starting material which has made it difficult to track.

The two vials that are enclosed represent 2-4 HPLC runs of the recrystallization solvent. They elute from a semiprep C_{18} column between 28 and 36 min (see the attached chromatogram) using a mobile phase of 30% methanol/70% water isocratically for 15 min followed by a linear gradient to 100 % methanol over 15 min. We have run our detector at lower wavelengths and find little difference in the profile.

Marvin and I very much appreciate your help with this project and hope that you are able to drag something out of this fraction. Please let me know if there is anything I can do to help.

Sincerely,

Alan Buckpitt, Ph.D.
Associate Professor
of Toxicology

cc: Dr. Marvin Goldman
Laboratory of Energy Related Health Research

(916) 752-1011
(916) 752-0721

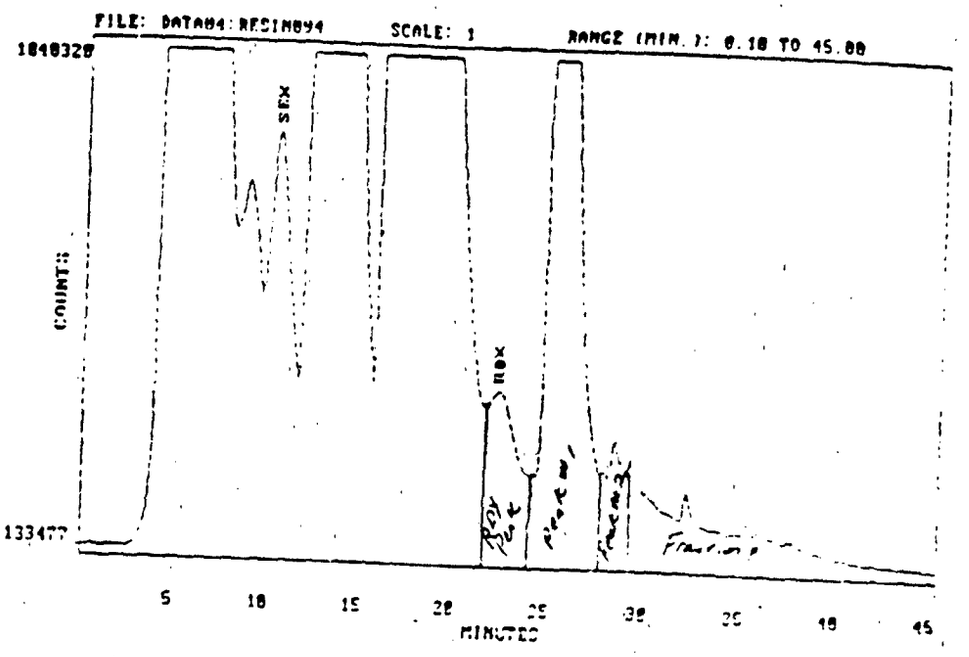

Figure 2. HPLC chromatographic profile (UV at 254 nm) of evaporator sludge indicating the fractions collected for assay using the Ames salmonella tester strain TA 1537.

RDX
HMX
TAX
SEX

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